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(54) Polypeptide, DNA fragment encoding the same, drug composition containing the same and process for producing the same.

(57) This invention particularly provides a novel polypeptide having high protease-inhibiting activity, preferably FXa-inhibiting activity, which comprises, at least as a part of the polypeptide, an amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the following amino acid sequence (1), wherein the amino acid substitution is at least one substitution selected from the following substitution means (i) to (iii). It also provides a process for the production of the polypeptide, a novel DNA fragment encoding the polypeptide and a drug composition containing the same.

Amino acid sequence (1)

Cys Asn Leu Pro Ile Val Arg Gly Pro Cys
Arg Ala Phe Ile Gln Leu Trp Ala Phe Asp
Ala Val Lys Gly Lys Cys Val Leu Phe Pro
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys
Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr
Cys

(i) Substitution of 15 position Gln counting from the N-terminus by an amino acid other than Gln.

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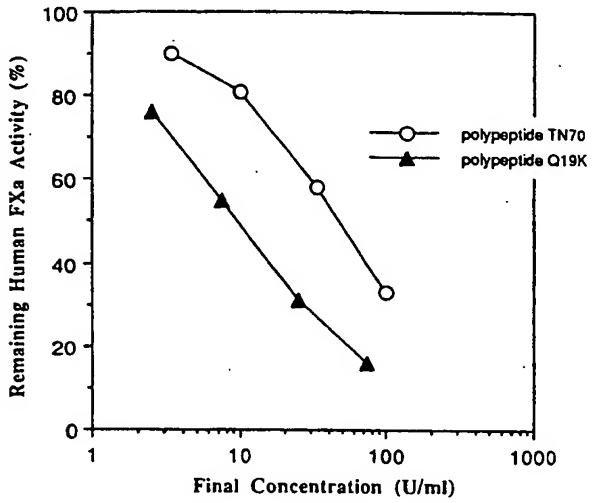
(ii) Substitution of 42 position Tyr counting from the N-terminus by an amino acid other than Tyr.
(iii) Substitution of 7 position Arg counting from the N-terminus by an amino acid other than Arg.

Fig. 46



FIELD OF THE INVENTION

This invention relates to a novel polypeptide, a novel DNA fragment encoding the novel polypeptide, a vector containing the novel DNA fragment, a transformant capable of producing the novel polypeptide, a process for producing the novel polypeptide, a drug composition containing the novel polypeptide as an effective ingredient and a protease inhibition process in which the novel polypeptide is used.

BACKGROUND OF THE INVENTION

As it is universally known, various proteases including trypsin and chymotrypsin exist in the body. While these proteases take important roles in the body such as digestion, defense mechanism, blood coagulation and fibrinolysis and the like, it has been revealed by previous studies that they also cause diseases and troubles directly or indirectly. Shock, pancreatitis, disseminated intravascular coagulation syndrome (DIC) and the like are known as typical diseases which are caused by abnormal activation of proteases.

Various types of protease inhibitors have been used with the aim of treating such protease-related diseases. The protease inhibitors so far used as pharmaceutical drugs for the treatment of these diseases are divided into two groups, namely chemically synthesized compounds and natural substances. In most cases, chemically synthesized compounds are applicable to oral administration and have a broad enzyme inhibition spectrum, while each of the natural substances inhibits each own specific protease and is possessed of other functions than its enzyme inhibition function, such as a cell growth enhancing activity and the like.

Inhibition spectrum of each natural protease inhibitor is greatly related to the kinds and sequence of amino acids on its active center. For example, trypsin type enzymes are inhibited by a protease inhibitor when an amino acid of a principal position P_1 on the active center of the protease inhibitor is Lys or Arg, chymotrypsin type enzymes are inhibited when the P_1 amino acid is Phe or Tyr and elastase type enzymes are inhibited when the P_1 amino acid is Ala, Ser or Val (Laskowski M., Jr., *Biochem. Pharmacol.*, vol.29, pp.2089 - 2094, 1980). In consequence, it is assumed that inhibition spectrum of a natural protease inhibitor can be changed by substituting an amino acid which is a composing element of the active center of the inhibitor. Such an approach has already been applied to several natural protease inhibitors. For example, Brinkmann *et al.* have reported that chymotrypsin inhibition activity of aprotinin was improved when positions P_1 and P'_2 on the active center of aprotinin were substituted by a hydrophobic amino acid such as Phe, Tyr, Leu or the like (Thomas Brinkmann *et al.*, *Eur. J. Biochem.*, vol.202, pp.95 - 99, 1991). In addition, Fritz *et al.* have prepared a substance by substituting an amino acid on the active center of Bikunins (HI-30) by other amino acid, and have measured its elastase- and trypsin-inhibiting activities (Japanese Patent Application Kokai No. 3-255099, 1991; European Patent EP401508, 1990).

Since changes in the properties of a protease inhibitor caused by such an amino acid substitution exert influence not only upon its enzyme inhibition spectrum but also upon its route of administration, pharmacological function and the like, it is important to develop various types of protease inhibitors having such new characteristics for use in the treatment of diseases and to use the new inhibitors according to the conditions of each disease to be treated.

SUMMARY OF THE INVENTION

In view of the above, it therefore becomes an object of the present invention to provide a novel polypeptide as a protease inhibitor having new characteristics as described above, especially having high FXa inhibition activity, which comprises, at least as a part of the polypeptide, an amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the following amino acid sequence (1), wherein the amino acid substitution is at least one substitution selected from the following substitution means (i) to (iii).

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Amino acid sequence (1)

Cys Asn Leu Pro Ile Val Arg Gly Pro Cys
Arg Ala Phe Ile Gln Leu Trp Ala Phe Asp
Ala Val Lys Gly Lys Cys Val Leu Phe Pro
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys

Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr
Cys

Substitution means

(i) Substitution of 15 position Gln counting from the N-terminus by an amino acid other than Gln.
(ii) Substitution of 42 position Tyr counting from the N-terminus by an amino acid other than Tyr.
(iii) Substitution of 7 position Arg counting from the N-terminus by an amino acid other than Arg.

Another object of the present invention is to provide a DNA fragment having a nucleotide sequence which encodes the novel polypeptide, a vector containing the DNA fragment, a transformant transformed with the DNA fragment or with the vector containing the DNA fragment and a process for producing the novel polypeptide.

A further object of the present invention is to provide a drug composition containing the novel polypeptide as an effective ingredient and a protease inhibition process in which the novel polypeptide is used.

Other objects and advantages of the present invention will be made apparent as the description progresses.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing nucleotide sequences of oligonucleotides S33, S34, S35, S18, S19 and S20.
Fig. 2 is a graph showing a process for the construction of plasmid pM468.
Fig. 3 is a graph showing a process for the construction of plasmid pM469.
Fig. 4 is a graph showing plasmid pM552.
Fig. 5 is a graph showing a nucleotide sequence of Y46E primer.
Fig. 6 is a graph showing a nucleotide sequence of *Sca*I sense primer.
Fig. 7 is a graph showing a nucleotide sequence of *Bam*HI primer.
Fig. 8 is a graph showing a process for the construction of plasmid pM575B.
Fig. 9 is a graph showing a nucleotide sequence of a region of plasmid pM575B from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.
Fig. 10 is a graph showing a nucleotide sequence of oligomer TV12DD.
Fig. 11 is a graph showing a nucleotide sequence of *Hind*III primer.
Fig. 12 is a graph showing a nucleotide sequence of Q19K primer.
Fig. 13 is a graph showing a nucleotide sequence of pBR *Bam*HI primer.
Fig. 14 is a graph showing a process for the construction of plasmid pM576.
Fig. 15 is a graph showing a process for the construction of plasmid pM576.
Fig. 16 is a graph showing a nucleotide sequence of a region of plasmid pM576 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.
Fig. 17 is a graph showing a nucleotide sequence of AN68 primer.
Fig. 18 is a graph showing a nucleotide sequence of *Sac*II primer.
Fig. 19 is a graph showing a nucleotide sequence of Q19R primer.
Fig. 20 is a graph showing a process for the construction of plasmid pM735.
Fig. 21 is a graph Showing a process for the construction of plasmid pM735.
Fig. 22 is a graph showing a nucleotide sequence of a region of plasmid pM735 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 23 is a graph showing a process for the construction of plasmid pM736.

Fig. 24 is a graph showing a nucleotide sequence of a region of plasmid pM736 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 25 is a graph showing a nucleotide sequence of R11E primer.

Fig. 26 is a graph showing a process for the construction of plasmid pM726B.

Fig. 27 is a graph showing a nucleotide sequence of a region of plasmid pM726B from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 28 is a graph showing a process for the construction of plasmid pM575C.

Fig. 29 is a graph showing a nucleotide sequence of a region of plasmid pM575C from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 30 is a graph showing a process for the construction of plasmid pM576B.

Fig. 31 is a graph showing a nucleotide sequence of a region of plasmid pM576B from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 32 is a graph showing a process for the construction of plasmid pM737B.

Fig. 33 is a graph showing a nucleotide sequence of a region of plasmid pM737B from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 34 is a graph showing a nucleotide sequence of Linker 710.

Fig. 35 is a graph showing a nucleotide sequence of Y46D primer.

Fig. 36 is a graph showing a nucleotide sequence of a region of plasmid pM727 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 37 is a graph showing a nucleotide sequence of a region of plasmid pM744 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 38 is a graph showing a nucleotide sequence of R11Q primer.

Fig. 39 is a graph showing a nucleotide sequence of a region of plasmid pM741 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 40 is a graph showing a nucleotide sequence of R11D primer.

Fig. 41 is a graph showing a nucleotide sequence of a region of plasmid pM742 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 42 is a graph showing a nucleotide sequence of R11L primer.

Fig. 43 is a graph showing a nucleotide sequence of a region of plasmid pM743 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 44 is a graph showing a nucleotide sequence of a region of plasmid pM738 from its *Hind*III site to *Bam*HI site, and a corresponding amino acid sequence.

Fig. 45 is a graph showing FXa-inhibiting activity of polypeptide Y46E of the present invention.

Fig. 46 is a graph showing FXa-inhibiting activity of polypeptide Q19K of the present invention.

Fig. 47 is a graph showing FXa-inhibiting activity of polypeptide Q19R of the present invention.

Fig. 48 is a graph showing FXa-inhibiting activity of polypeptide Q19K/Y46E of the present invention.

Fig. 49 is a graph showing FXa-inhibiting activity of polypeptide R11E/Y46E of the present invention.

Fig. 50 is a graph showing FXa-inhibiting activity of polypeptide Q19R/Y46E of the present invention..

Fig. 51 is a graph showing FXa-inhibiting activity of polypeptides Q19K/Y46D and Q19R/Y46D of the present invention.

Fig. 52 is a graph showing FXa-inhibiting activity of polypeptides R11Q/Q19K/Y46D, R11D/Q19K/Y46D and R11L/Q19K/Y46D of the present invention.

Fig. 53 is a graph showing FXa-inhibiting activity of polypeptide R11E/Q19K/Y46E of the present invention.

Fig. 54 is a graph showing elastase-inhibiting activity of polypeptide R11Q/Q19K/Y46D, R11D/Q19K/Y46D and R11L/Q19K/Y46D of the present invention.

Fig. 55 is a graph showing elastase-inhibiting activity of polypeptide R11E/Q19K/Y46E of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

With the aim of overcoming aforementioned problems involved in the prior art, the inventors of the present invention have conducted intensive studies on the development of a new protease inhibitor by introducing a mutation into a polypeptide which comprises an amino acid sequence represented by the following formula 1.

Formula 1

```
                    5                   10
Cys Asn Leu Pro Ile Val Arg Gly Pro Cys

                   15                   20
Arg Ala Phe Ile Gln Leu Trp Ala Phe Asp

                   25                   30
Ala Val Lys Gly Lys Cys Val Leu Phe Pro

                   35                   40
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys

                   45                   50
Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr

Cys
```

The polypeptide represented by the above amino acid sequence of formula 1 has been found for the first time by the present inventors as a polypeptide having an activity to inhibit activated blood coagulation factor X (to be referred to as "FXa" hereinafter) (Japanese Patent Application No. 03-325220). The amino acid sequence of this polypeptide coincides with a part of the amino acid sequence of urinary trypsin inhibitor (UTI) or Bikunins (HI-30). However, the polypeptide represented by the above amino acid sequence of formula 1 is clearly different from UTI or HI-30, because the polypeptide of formula 1 has a high FXa-inhibiting activity while the latter two peptides hardly show such an activity. The inventors of the present invention have conducted intensive studies with the aim of improving added value of the polypeptide of formula 1. As the results, the present inventors have succeeded in obtaining a novel polypeptide having new characteristics by substituting an amino acid at a specific position in the above amino acid sequence by other amino acid, a position which could not be predicted from the information so far available. In other words, the inventors of the present invention have succeeded in developing a novel polypeptide having new characteristics unknown in the prior art by substituting an amino acid at a specific position by other amino acid, a position which is different from the active center reported by Wachter et al. and by Sailer (Wachter *et al.*, *Hoppe-Seyler's Z. Physiol. Chem.*, vol.360, pp.1297 - 1303, 1979; and Jean-Philippe Sailer, *TIBS*, vol.15, pp.435 - 439, 1990).

According to a first aspect of the present invention, there is provided a novel polypeptide which contains, at least as a part thereof, an amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the aforementioned amino acid sequence represented by formula 1.

According to a second aspect of the present invention, there is provided a novel DNA fragment containing a nucleotide sequence which encodes the polypeptide of the first aspect of the present invention.

According to a third aspect of the present invention, there is provided a vector which contains the DNA fragment of the second aspect of the present invention.

According to a fourth aspect of the present invention, there is provided a transformant which is transformed with the DNA fragment of the second aspect of the present invention.

According to a fifth aspect of the present invention, there is provided a transformant which is transformed with the vector of the third aspect of the present invention.

According to a sixth aspect of the present invention, there is provided a process for producing the polypeptide of the first aspect of the present invention, which comprises the following order of steps of:

(a) preparing a DNA fragment containing a nucleotide sequence which encodes the polypeptide of the first aspect of the present invention,

(b) isolating a transformant by transforming a host cell with the DNA fragment prepared in the above step (a), and

(c) culturing the transformant obtained in the above step b) thereby allowing the transformant to produce the polypeptide of the first aspect of the present invention and subsequently recovering said polypeptide from cultured medium.

According to a seventh aspect of the present invention, there is provided a process for producing the polypeptide of the first aspect of the present invention, which comprises th following order of steps of:

(a) preparing a DNA fragment containing a nucleotide sequence which encodes the polyp ptide of the first aspect of the present invention,

(b) preparing a vector which contains the DNA fragment obtained in the above step (a),

(c) isolating a transformant by transforming a host cell with the vector obtained in the above step b), and

(d) culturing the transformant obtained in the above step c) thereby allowing it to produce a polypeptide of the first aspect of the present invention and subsequently recovering said polypeptide from cultured medium.

According to an eighth aspect of the present invention, there is provided a drug composition which contains the polypeptide of the first aspect of the present invention as an active ingredient.

According to a ninth aspect of the present invention, there is provided a protease inhibition process which comprises using the polypeptide of the first aspect of the present invention.

The following describes the present invention in detail.

The novel polypeptide of the first aspect of the present invention is characterized in that it contains, at least as a part thereof, an amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the aforementioned amino acid sequence represented by formula 1.

The term "a polypeptide containing an amino acid sequence as a part thereof" as used herein means that, when a polypeptide is defined by its primary structure, the polypeptide may be defined either by the amino acid sequence itself or by an amino acid sequence in which the N-terminus, the C-terminus or both termini of the former amino acid sequence is or are supplemented with one or more optional amino acids. Polypeptide-defining elements other than the primary structure, such as the presence of a sugar chain and the like, are not particularly limited.

Preferably, substitution of an amino acid may be effected at least at 7 position, 15 position or 42 position of the amino acid sequence of the aforementioned formula 1, counting from its N-terminus. In other words, an amino acid sequence to be used at least as a part of the novel polypeptide of the first aspect of the present invention may be derived from the amino acid sequence of formula 1 by substituting only Arg at the 7 position of the sequence by other amino acid, only Gln at the 15 position by other amino acid or only Tyr at the 42 position by other amino acid, counting from the N-terminus of the amino acid sequence. Also, at least two of the 7 position Arg, 15 position Gln and 42 position Tyr may be substituted simultaneously by other amino acids. In addition to these substitutions, one or more other amino acids at different positions may be substituted by optional amino acids.

Preferably, the substitution of the amino acids may at least increase the activity to inhibit protease or improve the secretion of the polypeptide from host cells when the polypeptide is produced by recombinant DNA techniques in comparison with the original polypeptide prior to the amino acid substitution.

More preferably, the substitution of the amino acids may be effective for improving at least one property of the polypeptide selected from the group consisting of;

(i) increasing the activities of the polypeptide to inhibit FXa,

(ii) having an improved secretion from a transformant when the polypeptide is produced by recombinant DNA techniques, and

(iii) increasing the activities of the polypeptide to inhibit elastase.

Substitution of the 15 position Gln or 42 position Tyr by other amino acid is effective for changing activities to inhibit protease of polypeptide which contains the amino acid sequence of formula 1, especially for its changing activities to inhibit at least FXa. In addition, substitution of the 7 position Arg by other amino acid is effective for changing its activities to inhibit protease, especially at least elastase.

Substitution of the 7 position Arg by other amino acid is effective for easy secretion of the resulting polypeptide from host cells when the polypeptide is produced by recombinant DNA techniques. When at least two of the 7 position, 15 position and 42 position amino acids are substituted simultaneously by other amino acids, it is possible to obtain additive effects of the employed substitutions.

The polypeptide of the first aspect of the present invention may preferably contain, at least as a part thereof, an amino acid sequence resulting from at least one substitution in the amino acid sequence of formula 1, which is selected from the following substitutions (1) to (11);

(1) substitution of Lys for the 15 position Gln counting from the N-terminus,

(2) substitution of Arg for the 15 position Gln counting from the N-terminus,

(3) substitution of Glu for the 42 position Tyr counting from the N-terminus,

(4) substitution of Asp for the 42 position Tyr counting from the N-terminus,

(5) substitution of Glu for the 7 position Arg counting from the N-terminus,

(6) substitution of Gln for the 7 position Arg counting from the N-terminus,

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(7) substitution of Asp for the 7 position Arg counting from the N-terminus,
(8) substitution of Leu for the 7 position Arg counting from the N-terminus,
(9) substitution of Asn for the 7 position Arg counting from the N-terminus,
(10) substitution of Ser for the 7 position Arg counting from the N-terminus, and
(11) substitution of Ala for the 7 position Arg counting from the N-terminus.

Aforementioned effects of the substitution of at least one amino acid in the amino acid sequence of formula 1 on the properties of the resulting polypeptide can also be found when the N-terminus, the C-terminus or both termini of the formula 1 sequence is or are supplemented with one or more optional amino acids. In consequence, the novel polypeptide of the present invention may contain, at least as a part thereof, an amino acid sequence in which the N-terminus, the C-terminus or both termini of the amino acid sequence resulting from substitution of at least one amino acid in the amino acid sequence of formula 1 by other amino acid is or are further added with one or more optional amino acids. Kinds and numbers of amino acids to be added are not particularly limited, provided that the characteristic nature of the novel polypeptide of the present invention is not spoiled completely by the amino acid addition.

A preferred amino acid sequence to be added to the N-terminus may be selected from the following sequences (1) to (5);

(1) Asp Asp Ala Ala,

(2) Thr Val Ala Ala,

(3) Val Ala Ala,

(4) Ala Ala, and

(5) Ala.

A derivative of each of the above amino acid sequences (1) to (5) may also be used as an amino acid sequence to be added to the N-terminus, such as a derivative in which at least one amino acid in any of the amino acid sequences (1) to (5) is substituted by other optional amino acid, a derivative in which at least one optional amino acid is added to or deleted from any of the amino acid sequences (1) to (5) or a derivative in which such substitution, addition and deletion are effected simultaneously in any of the amino acid sequences (1) to (5). These added sequences are useful when the novel polypeptide is produced using an *Escherichia coli*.

A preferred amino acid sequence to be added to the C-terminus may be selected from the following sequences (1) to (15);

(1) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn,

(2) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe Ser,

(3) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe,

(4) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg,

(5) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu,

(6) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu,

(7) Gly Val Pro Gly Asp Gly Asp Glu Glu,

(8) Gly Val Pro Gly Asp Gly Asp Glu,

(9) Gly Val Pro Gly Asp Gly Asp,

(10) Gly Val Pro Gly Asp Gly,

(11) Gly Val Pro Gly Asp,

(12) Gly Val Pro Gly,

(13) Gly Val Pro,

(14) Gly Val, and

(15) Gly.

A derivative of each of the above amino acid sequences (1) to (15) may also be used as an amino acid sequence to be added to the C-terminus side, such as a derivative in which at least one amino acid in any of the amino acid sequences (1) to (15) is substituted by other optional amino acid, a derivative in which at least one optional amino acid is added to or deleted from any of the amino acid sequences (1) to (15) or a derivative in which such substitution, addition and deletion are effected simultaneously in any of the amino acid sequences (1) to (15). These sequences are useful when the novel polypeptide is produced using an *Escherichia coli* strain.

It goes without saying that the above-mentioned preferred amino acid sequences may be added to each N-terminus , C-terminus or both termini of N- and C-termini.

Most preferred examples of the amino acid sequence of the novel polypeptide of the first aspect of the present invention are shown in the SEQUENCE LISTING attached hereto as Sequence ID Nos. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 29, 30 and 31.

The novel polypeptide according to the first aspect of the present invention may have a sugar chain.

Recent advance in the technology has rendered possible application of various chemical modifications to polypeptides, such as alkylation, oxidation, reduction, hydrolysis and the like. Also, formation of a salt with a pharmacologically acceptable acid or base and linkage of polyethylene glycol or the like to a polypeptide from a DDS (drug delivery system) point of view are usually used techniques. In consequence, polypeptides to which such modifications are applied are also included in the novel polypeptide of the present invention.

Preferably, the novel polypeptide of the first aspect of the present invention may have at least a protease inhibition activity as one of its characteristic properties. The protease inhibition activity may preferably be at least one inhibitional activities to FXa, elastase and trypsin.

The novel polypeptide according to the first aspect of the present invention may be obtained by any means. For instance, it may be obtained by chemical synthesis using a peptide synthesizer (for example, model 431 manufactured by Applied Biosystems). It may be obtained also by known recombinant DNA

techniques disclosed for instance by T. Maniatis *et al.* in *Molecular Cloning, a laboratory manual* (Cold Spring Harbor Laboratory, 1982), using a DNA fragment which encodes the novel polypeptide of the present invention. A preferred example of th production of the novel polypeptide of the present invention by means of recombinant DNA techniques will be described later in relation to the sixth and seventh aspect of the present invention.

Next, the novel DNA fragment of the second aspect of the present invention is described.

The novel DNA fragment of the present invention contains, at least as a part thereof, a nucleotide sequence which encodes the novel polypeptide of the first aspect of the present invention. In other words, the novel DNA fragment of the present invention is characterized in that it comprises a nucleotide sequence which encodes a polypeptide that contains, at least as a part thereof, an amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the aforementioned amino acid sequence of formula 1.

The term "a DNA fragment containing a nucleotide sequence as a part thereof" as used herein means that the DNA fragment may be defined either by the nucleotide sequence itself or by a nucleotide sequence in which the 5' end, the 3' end or both ends of the former nucleotide sequence is or are supplemented with one or more optional nucleotides. The novel DNA fragment of the present invention may have any nucleotide sequence, provided that the novel polypeptide of the first aspect of the present invention is produced in appropriate host cells which have been transformed with the DNA fragment by an appropriate means. Since a degeneracy of codons exist corresponding to one amino acid as it is universally known, the nucleotide sequence which encodes the novel polypeptide of the first aspect of the present invention is not limited to a single kind and, therefore, the nucleotide sequence of the novel DNA fragment of the present invention is also not limited to a single kind. However, a nucleotide sequence which encodes the aforementioned amino acid sequence of formula 1 may preferably have the following formula 2.

Formula 2

```
1           10           20           30
TGC AAT CTC CCC ATA GTC CGG GGC CCC TGC

            40           50           60
CGA GCC TTC ATC CAG CTC TGG GCA TTT GAT

            70           80           90
GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC

           100          110          120
TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG

           130          140          150
TTC TAC TCA GAG AAG GAG TGC AGA GAG TAC


TGC
```

Preferably, the novel DNA fragment of the present invention is a DNA fragment contains, at least as a part thereof, a nucleotide sequence in which one or more nucleotides in the nucleotide sequence of the above formula 2 are substituted by other nucleotides selected from adenine (A), guanine (G), thymidine (T) and cytosine (C).

When the amino acid sequence of the polypeptide of the first aspect of the present invention is taken into consideration, it is preferable to effect the nucleotide substitution in the aforementioned formula 2 at a 19 to 21 position nucleotide sequence, at a 43 to 45 position nucleotide sequence or at a 124 to 126 position nucleotide sequence, counting from the 5' end of the formula 2 nucleotide sequence. In other words, in the nucleotide sequence which is contained at least as a part of the novel DNA fragment the second aspect of the present invention, the nucleotide substitution by other nucleotides may be effected only at a 19 to 21 position nucleotide sequence CGG, at a 43 to 45 position nucleotide sequence CAG or at a 124 to 126 position nucleotide sequence TAC, counting from the 5' end of the nucleotide sequence of

formula 2. Also, the nucleotide substitution may be effected by other nucl otides at least two of these positions. In addition to these nucleotide sequence substitutions, one or more other nucleotides at different positions may be substituted by optional nucleotides.

The aforementioned 19 to 21 position nucleotide sequence CGG which encodes Arg may be substituted by an optional nucleotide sequence, preferably by a nucleotide sequence that encodes Glu, Gln, Asp, Leu, Asn, Ser or Ala. The 43 to 45 position nucleotide sequence CAG which encodes Gln may be substituted by an optional nucleotide sequence, preferably by a nucleotide sequence that encodes Lys or Arg. The 124 to 126 position nucleotide sequence TAC which encodes Tyr may be substituted by an optional nucleotide sequence, preferably by a nucleotide sequence that encodes Glu or Asp.

More preferably, the novel DNA fragment of the second aspect of the present invention may contain, at least as a part thereof, a derivative of the nucleotide sequence of formula 2 resulting from at least one substitution selected from the following substitutions (1) to (11);

(1) substitution of AAG for the 43 to 45 position nucleotide sequence CAG counting from the 5' end,
(2) substitution of CGT for the 43 to 45 position nucleotide sequence CAG counting from the 5' end,
(3) substitution of GAA for the 124 to 126 position nucleotide sequence TAC counting from the 5' end,
(4) substitution of GAC for the 124 to 126 position nucleotide sequence TAC counting from the 5' end,
(5) substitution of GAA for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(6) substitution of CAG for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(7) substitution of GAT for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(8) substitution of CTG for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(9) substitution of AAC for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(10) substitution of AGC for the 19 to 21 position nucleotide sequence CGG counting from the 5' end, and
(11) substitution of GCG for the 19 to 21 position nucleotide sequence CGG counting from the 5' end.

The DNA fragment of the present invention may contain, at least as a part thereof, a nucleotide sequence in which one or more optional nucleotides are added to its 5' end, 3' end or both ends of the nucleotide sequence resulting from the substitution of at least one nucleotide in the nucleotide sequence of formula 2. Kinds and numbers of the nucleotides to be added are not limited, provided that the finally obtained DNA fragment contains a nucleotide sequence which encodes the polypeptide of the first aspect of the present invention. For example, the 5' end may be supplemented with an initiation codon, a promoter, a ribosome binding region, a signal peptide-encoding sequence or the like, and the 3' end may be supplemented with a termination codon. In addition, a nucleotide sequence which is recognized by an appropriate restriction enzyme may be added to the 5' end and/or the 3' end, or a nucleotide sequence which encodes other polypeptide may be added to the 5' end and/or the 3' end with the aim of producing the novel polypeptide of the first aspect of the present invention in the form of a fused protein with other polypeptide.

Preferred examples of the nucleotide sequence to be added to the 5' end include those which encode the amino acid sequences described as "preferred amino acid sequences to be added to the N-terminus" in the foregoing description about the polypeptide of the first aspect of the present invention, or which encode derivatives of these amino acid sequences resulting from deletion, addition, substitution and the like of one or more amino acids. Any nucleotide sequence which encodes such an amino acid sequence may be added to the 5' end as a preferred example, but a more preferred example may be selected from the following sequences (1) to (5).

(1) GAC GAC GCC GCC

(2) ACC GTC GCC GCC

(3) GTC GCC GCC

(4) GCC GCC

(5) GCC

Preferred examples of the nucleotide sequence to be added to the 3' end include those which encode the amino acid sequences described as "preferred amino acid sequences to be added to the C-terminus" in the foregoing description about the polypeptide of the first aspect of the present invention, or which

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encode derivatives of th se amino acid sequences resulting from deletion, addition, substitution and the like of one or more amino acids. Any nucleotide sequence which ncodes such an amino acid sequence may be added to the 3' end as a pref rr d example, but a more preferred illustrative example may be selected from the following sequences (1) to (15);

(1) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC TCC AAC,

(2) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC TCC,

(3) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC,

(4) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC,

(5) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG,

(6) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG,

(7) GGT GTC CCT GGT GAT GGT GAT GAG GAG,

(8) GGT GTC CCT GGT GAT GGT GAT GAG,

(9) GGT GTC CCT GGT GAT GGT GAT,

(10) GGT GTC CCT GGT GAT GGT,

(11) GGT GTC CCT GGT GAT,

(12) GGT GTC CCT GGT,

(13) GGT GTC CCT,

(14) GGT GTC, and

(15) GGT.

It goes without saying that the above-mentioned preferred DNA sequences may be added to each 5' end, 3' end or both ends of 5' and 3' ends.

Most preferred examples of the nucleotide sequence of the novel DNA fragment of the second aspect of the present invention are shown in the SEQUENCE LISTING attached hereto as Sequence ID Nos. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 32, 33 and 34.

The novel DNA fragment of the present invention may be obtained by any means. For instance, it may be obtained by chemical synthesis or by recombinant DNA techniques. Chemical synthesis of the novel DNA fragment of the present invention may be effected for example in the following manner. Firstly, a desired nucleotide sequence is designed and the thus designed DNA fragment is divided into appropriate size of small fragments. An oligomer which corresponds to each of the thus divided fragments is synthesized using an fully automatic DNA synthesizer (for example, model 394 manufactured by Applied Biosystems). The thus synthesized oligomer is subjected to annealing. If necessary, 5'-end phosphoriza-tion is carried out using T4 polynucleotide kinase prior to the annealing step. Thereafter, the thus annealed fragments are subjected if necessary to ligation with T4 DNA ligase, and then cloned into an appropriate vector.

When the novel DNA fragment, of the present invention is prepared by means of recombinant DNA techniques, it may be effected by nucleotide sequence modification and DNA amplification making use of an appropriate cDNA library, a chromosomal DNA library or a DNA fragment which encodes the amino acid sequence of formula 1, in accordance with usually used techniques such as sit -directed mutagenesis (Kramer, W. *et al.*, *Nucleic Acid Res.*, vol.12, pp.9441 - 9456. 1984; Kunkel, T.A. *et al.*, *Methods in*

Enzymology, vol.154, pp.357 - 382, 1987) and polymerase chain reaction (to be refereed "PCR" hereinafter) (*PCR Protocols, A Guide to Methods and Applications*, edited by Michael,A.I. et al., Academic Press, 1990).

A cDNA library or a chromosomal DNA library to be used may be chosen from commercially available articles, or prepared from appropriate tissues or cells according to usually used means (cf. *Molecular Cloning, A Laboratory Manual*, edited by T. Maniatis *et al.*, Cold Spring Harbor Laboratory, 1982). A DNA fragment which encodes the amino acid sequence of formula 1 may be obtained not only by chemical synthesis but also from an appropriate DNA library optionally making use of known recombinant DNA techniques such as hybridization (cf. Wallace, R.B. *et al.*, *Nucleic Acid Res.*, vol.9, pp.879 - 894, 1981).

On the basis of the disclosure of the novel DNA fragment of the present invention, its complementary DNA fragments and RNA fragments can also be obtained. The DNA fragment of the present invention may also include the linkage by such a complementary DNA or RNA fragment.

According to the third aspect of the present invention, there is provided a vector which contains the novel DNA fragment of the second aspect of the present invention.

In addition to the DNA fragment of the second aspect of the present invention, the vector of the present invention may further contain additional nucleotide sequences such as of a promoter, a ribosome binding region, a signal peptide-encoding sequence, a selection marker sequence and the like, as well as a sequence which is used to amplify copy numbers of the DNA of interest. The vector of the present invention may be used for any purpose. For example, it may be used as an expression vector or as a cloning vector.

Vectors for expression use generally has a promoter, an optional ribosome binding region and the like sequences which are necessary for the expression. In the vector of the present invention, these necessary sequences are used by selecting them in such a way that they can function in host cells to be transformed with the vector. When a polypeptide expressed in the cells of a transformant is secreted outside the cells, a vector may generally contain a nucleotide sequence which encodes a signal peptide in addition to the above necessary sequences. In consequence, when the vector of the third aspect of the present invention is used as an expression vector, it may contain not only the DNA fragment of the second aspect of the present invention but also these nucleotide sequences necessary for expression and, if required, a nucleotide sequence which encodes a signal peptide.

The vector of the third aspect of the present invention may be obtained by inserting the DNA fragment of the second aspect of the present invention into an appropriate commercially available or known vector in accordance with usually used means (cf. *Molecular Cloning, A Laboratory Manual*, edited by T. Maniatis *et al.*, Cold Spring Harbor Laboratory, 1982). The origin of the vector to be used for the insertion of the DNA fragment of the second aspect of the present invention is not particularly limited. Such a vector may be selected from various virus vectors, plasmid vectors, phage vectors and the like, such as pUC118, pBR322, pEFN-BOS, λgt10, pKK223-3, YAC, Baculo virus vector and the like. If necessary, optional nucleotide sequences such as a ribosome binding region, a selection marker sequence and the like may be synthesized chemically and inserted together with the DNA fragment of the second aspect of the present invention. Alternatively, the DNA fragment of the second aspect of the present invention may be inserted into a commercially available vector which already contains these desired nucleotide sequences.

Most preferred vector of the present invention is a plasmid vector which contains at least a tryptophan promoter, a kanamycin or ampicillin resistant gene and the DNA fragment of the second aspect of the present invention.

According to the fourth aspect of the present invention, there is provided a transformant transformed with the novel DNA fragment of the second aspect of the present invention. A transformant which can produce the novel polypeptide of the first aspect of the present invention is preferable as the transformant of the fourth aspect of the present invention. Either transformant which secrets the polypeptide outside the cell or which accumulates the polypeptide in the cell is preferable as the transformant of the fourth aspect of the present invention.

The transformant of the fourth aspect of the present invention may be obtained by introducing the novel DNA fragment of the second aspect of the present invention into appropriate host cells in accordance with a usually used means such as calcium chloride technique, a method in which a calcium phosphate-DNA complex is used, microinjection technique, electroporation or the like.

Preferably, in addition to a nucleotide sequence encoding the novel polypeptide of the first aspect of the present invention, the novel DNA fragment of the second aspect of the present invention to be used for the preparation of the transformant may contain additional nucleotide sequences which encodes a promoter, a ribosome binding region and the like that are necessary for the expression of the novel polypeptide of the first aspect of the present invention. If necessary, the DNA fragment to be used may contain a nucleotide

sequenc which encodes a signal peptide in addition to oth r necessary nucl otide sequences for the expression of the polypeptide. These promoter, ribosome binding region, signal peptide-encoding sequence and the like may be of various origins, provid d that they can function in the host cells to be used.

Host cells to be used for the insertion of the novel DNA fragment of the second aspect of the present invention are not strictly limited, provided that they are suitable for use in the expression of the novel polypeptide of the first aspect of the present invention. Such suitable host cells may be selected from either eukaryotic cells such as HeLa cells, Namalwa cells, COS cells, CHO cells, yeast cells, SF cells and the like or prokaryotic cells such as *E. coli* cells, *Bacillus subtilis* cells and the like.

According to the fifth aspect of the present invention, there is provide a transformant transformed with the vector of the third aspect of the present invention. A transformant which can produce the novel polypeptide of the present invention is preferable as the transformant of the fifth aspect of the present invention. Either transformant which secrets the polypeptide outside the cell or which accumulates the polypeptide in the cell is preferable as the transformant of the fifth aspect of the present invention.

The transformant of the fifth aspect of the present invention is obtained by transforming an appropriate host cells with the vector of the third aspect of the present invention by a usually used means such as calcium chloride technique, rubidium chloride technique, Hanahan's method (Hanahan,D., Techniques for Transformation of *E. coli*; in *DNA Cloning*, vol.1, Glover,D.M. (ed.), pp.109 - 136, IRL Press, 1985) or the like.

Host cells to be used for the transformation by the vector of the third aspect of the present invention are not strictly limited, provided that they are suitable for use in the expression of the novel polypeptide of the first aspect of the present invention. Such suitable host cells may be selected from either eukaryotic cells such as HeLa cells, Namalwa cells, COS cells, CHO cells, yeast cells, SF cells and the like or prokaryotic cells such as *E. coli* cells, *B. subtilis* cells and the like.

As it is universally known, a host cell and a vector function mutually. Taking this into consideration, it is preferable to obtain the inventive transformant by selecting an appropriate origin of the vector, a nucleotide sequence necessary for expression in the vector and an appropriate host in such a combination that the novel polypeptide of the first aspect of the present invention can be expressed efficiently.

Illustrative examples of the combination of expression vector with host cells include: an expression vector containing the early promoter gene of simian virus 40 (SV40) with COS-7 cells; and an expression vector originated from plasmid pBR322 containing nucleotide sequences which encode a tryptophan promoter and a tryptophan SD sequence with *E. coli* HB101 cells.

As will be described later in Examples, the present inventors have prepared *E. coli* JE5505 transformants transformed with the vector of the third aspect of the present invention. These transformants have been deposited by the present inventors in the Patent Microorganisms Depositary Center of Fermentation Research Institute, Agency of Industrial Science and Technology (1-1-1, Higashi, Yatabecho, Tsukuba-gun, Ibaraki, Japan). The following shows deposition numbers and names of these transformants.

Date deposited	Deposition No.	Name
September 11, 1990	FERM P-11726	*E. coli* JE5505 (pM552)
September 10, 1991	FERM BP-3561	
	(transferred from FERM P-11726)	
July 16, 1991	FERM P-12357	*E. coli* JE5505 (pM575B)
October 17, 1991	FERM BP-3613	
	(transferred from FERM P-12357)	
July 16, 1991	FERM P-12358	*E. coli* JE5505 (pM576)
October 17, 1991	FERM BP-3614	
	(transferred from FERM P-12358)	
May 1, 1992	FERM P-12945	*E. coli* JE5505 (pM735)
May 1, 1992	FERM P-12946	*E. coli* JE5505 (pM736)
October 21, 1992	FERM BP-4041	*E. coli* JE5505 (pM741)

The following describes sixth and seventh aspects of the present invention, concerning processes suitable for the production of the novel polypeptide of the first aspect of the present invention, to which recombinant DNA techniques are applied.

According to the sixth aspect of the present invention, there is provided a process for the production of the novel polypeptide of the first aspect of the present invention which comprises the steps of:

a) preparing a DNA fragment containing a nucleotide sequence which encodes the novel polypeptide of the first aspect of the present invention,

b) preparing a transformant by transforming host cells with the DNA fragment obtained in the above step a), and

c) culturing the transformant obtained in the above step b) to allow the transformant to produce the novel polypeptide of the first aspect of the present invention and subsequently recovering said polypeptide from the cultured medium.

In the above step a), the term "a DNA fragment containing a nucleotide sequence which encodes the novel polypeptide of the first aspect of the present invention" preferably means a DNA fragment of the second aspect of the present invention. Preferably, this DNA fragment may further contain a nucleotide sequence which is necessary for the expression of the novel polypeptide of the first aspect of the present invention, in addition to a nucleotide sequence that encodes said novel polypeptide. If necessary, said DNA fragment may also contain a nucleotide sequence which encodes a signal peptide.

The transformant obtained in the above step b) belongs preferably to the transformant of the fourth aspect of the present invention, and it can therefore be obtained by the process described in the foregoing in relation to the fourth aspect of the present invention.

The thus obtained transformant in the above step b) is used in the subsequent step c). Culturing of the transformant may be effected by generally used means for the culturing of microorganisms or mammalian cells, in accordance with the procedure disclosed for instance in *Seibutsu Kagaku Kogaku* (or Biochemical Engineering; S. Aiba *et al.*, 1976, Tokyo University Press) or in *Soshiki Baiyo* (or Tissue Culture; J. Nakai *et al.*, 1976, Asakura Shoten).

Next, the novel polypeptide of the first aspect of the present invention thus produced by the transformant is recovered from the cultured medium of said transformant. In this instance, the thus produced novel polypeptide of the first aspect of the present invention may be isolated preferably from the cultured cells of the transformant when the product is not secreted extracellularly, or from culture supernatant when secreted into the medium. Purification and recovery of the novel polypeptide of the first aspect of the present invention from a cultured broth containing said polypeptide may be carried out in the light of various generally used means for the purification of polypeptides which have been disclosed in many reports and books such as *Seikagaku Jikken Koza* (or Biochemical Experiments; vol.I, Protein Chemistry, 1976, edited by The Japanese Biochemical Society, Tokyo Kagaku Dojin). When the polypeptide of the present invention forms an inclusion body inside the transformant cells, it is preferably to carry out solubilization, denaturation and refolding in its purification (cf. Thomas E. Creighton, *J. Mol. Biol.*, vol.87, pp.563 - 577, 1974).

Examples of protein purification techniques include ammonium sulfate precipitation, ultrafiltration, isoelectric precipitation, gel filtration, ion exchange chromatography, various affinity chromatographic techniques such as hydrophobic chromatography, antibody chromatography and the like, chromatofocusing, absorption chromatography and reverse phase chromatography. Consequently, recovery and purification of the novel polypeptide of the first aspect of the present invention may be carried out by selecting suitable ones from these techniques and, if necessary, making use of an HPLC system and the like, in appropriate order of the techniques.

According to the seventh aspect of the present invention, there is provided a process for the production of the novel polypeptide of the first aspect of the present invention which comprises the steps of:

a) preparing a DNA fragment containing a nucleotide sequence which encodes the novel polypeptide of the first aspect of the present invention,

b) constructing a vector containing the DNA fragment obtained in the above step a)

c) preparing a transformant by transforming host cells with the vector obtained in the above step b), and

d) culturing the transformant obtained in the above step c) to allow the transformant to produce the novel polypeptide of the first aspect of the present invention and subsequently recovering said polypeptide from the cultured medium.

In the above step a), the term "a DNA fragment containing a nucleotide sequence which encodes the novel polypeptide of the first aspect of the present invention" preferably means a DNA fragment of the second aspect of the present invention.

The vector obtained in the above step b) is preferably the vector of the third aspect of the present invention. More preferably, the vector obtained in the above step b) is a vector which further contains a nucleotide sequence necessary for the expression of the polypeptide of the first aspect of the present invention, in addition to a nucleotide sequence that encodes said polypeptide. If necessary, said vector may also contain a nucleotide sequence which encodes a signal peptide. Process for the construction of such a vector has already been described in the foregoing in relation to the third aspect of the present invention.

The transformant thus obtained in the above step c) belongs preferably to the transformant of the fifth aspect of the present invention, and it can therefore be obtained by the process described in the foregoing in relation to the fifth aspect of the present invention.

The thus obtained transformant in the above step c) is used in the subsequent step d) . Processes for the culturing of the transformant and subsequent recovery and purification of the novel polypeptide of the present invention from the cultured medium are the same as those described in the foregoing in relation to the sixth aspect of the present invention.

Thus, processes for the production of the novel polypeptide have been described with respect to the sixth and seventh aspects of the present invention. In the practice of these processes, a DNA fragment which contains a fused nucleotide sequence consisting of a nucleotide sequence coding for the polypeptide of the first aspect of the present invention and another nucleotide sequence encoding other polypeptide (*E. coli* β-galactosidase for example) or a part thereof may be used as the DNA fragment that contains a nucleotide sequence encoding the novel polypeptide of the first aspect of the present invention. In such an instance, a transformant transformed with said DNA fragment or with a vector prepared therefrom will produce a fused polypeptide consisting of the novel polypeptide of the present invention and other polypeptide (*E. coli* β-galactosidase for example). In that case, it is possible to obtain the novel polypeptide of the first aspect of the present invention by recovering said fused polypeptide and then treating it with appropriate chemical compounds, enzymes and the like to cut out and remove the additional polypeptide portion, followed by purification if necessary.

Next, the drug composition of the eighth aspect of the present invention is described.

The drug composition provided by the present invention contains the novel polypeptide of the first aspect of the present invention as an active ingredient. Single polypeptide or a plurality of polypeptides may be contained in the drug composition, provided that they belong to the novel polypeptide of the first aspect of the present invention.

Though it is possible to provide the medical field with fully effective drug composition of the present invention even if the drug composition of the eighth aspect of the present invention is composed solely of the novel polypeptide of the first aspect of the present invention (for example, an article of the polypeptide processed through pharmaceutically necessary steps such as lyophilizing, sterile filtration and the like), the drug composition may further contain pharmaceutically acceptable auxiliary components in a pharmaceutically acceptable amount.

Such auxiliary components to be included in the drug composition of the present invention are a base, a stabilizer, an antiseptic agent, preservation agent, an emulsifying agent, a suspending agent, a solvent, a solubilizing agent, a lubricant, a corrective agent, a coloring agent, an aromatic agent, a soothing agent, an excipient, a binder, a thickening agent, a buffer and the like. Illustrative examples of these auxiliary components include calcium carbonate, lactose, sucrose, sorbitol, mannitol, starch, amylopectin, cellulose derivatives, gelatin, cacao butter, distilled water for injection use, sodium chloride solution, Ringer solution, glucose solution, human serum albumin (HSA) and the like. Of these components, gelatin and albumin are especially useful as protein stabilizers. The auxiliary components to be added to the drug composition of the present invention may be selected in the light of "A List of Pharmaceutical Additives" (published by the Committee on Pharmaceutical Affairs Law, Tokyo Drug Industry Association and the Committee on Pharmaceutical Affairs Law Research, Osaka Drug Association) and decided depending on the dosage form and the like of the drug composition.

Dosage forms of the drug composition of the present invention are not particularly limited and can be selected according to each application purpose from for example injections, tablets, capsules, pills, granules, suppositories, solutions, suspensions, emulsions, powders, ointments, creams, gels, cataplasmas, lotions and the like.

Dose of the drug composition of the present invention may vary depending on th cont nt of active ingredient and conditions, age, sex, weight and the like of each patient to be treated. Preferably, however, the drug composition of the present invention may be administered in an amount of from 0.1 to 1,000 mg/kg, more preferably from 0.2 to 50 mg/kg, most preferably from 0.2 to 20 mg/kg, as the amount of the active ag nt. The drug composition of the present invention can be administered by various means depending on the conditions of each patient, such as oral administration, intramuscular injection, in-

traperitoneal injection, intradermal injection, subcutaneous injection, intravenous injection, intraarterial administration, rectal administration, vaginal administration and the like, as well as airway inhalation of the drug composition making it into an aerosol dosage form, buccal dissolution, percutaneous absorption, mucosal absorption and the like, of which intravenous injection is particularly preferred.

Diseases and symptoms which may be prevented and/or treated by making use of the drug composition of the present invention include infestation, multiple organ failure, shock, pancreatitis, disseminated intravascular coagulation syndrome, ischemic heart disease, nephritis, hepatic cirrhosis, re-obstruction at the time of blood circulation reconstructive operation, edema caused by increased vascular permeability, adult respiratory distress syndrome, rheumatoid arthritis, arthritis, allergic diseases and the like.

Application of the drug composition of the present invention is not limited to the prevention and/or treatment of specific diseases. It can be used suitably as a blood coagulation inhibitor. Also, in addition to its direct administration to the body, the inventive drug composition can be used for the purpose of preventing blood coagulation, by binding and adsorbing it to the surface of medical devices such as artificial blood vessel, artificial organs, catheters and the like making use of a cross linking agent and the like.

Next, the ninth aspect of the present invention is described.

The protease inhibition process of the ninth aspect of the present invention is characterized in that it uses the novel polypeptide of the first aspect of the present invention. More particularly, the ninth aspect of the present invention is a process for inhibiting the activity of a protease which comprises allowing the novel polypeptide of the first aspect of the present invention to react with said protease.

The protease to be inhibited in the protease inhibition process of the present invention is not particularly limited and may be a single protease or a plurality of proteases, provided that they are sensitive to the novel polypeptide of the first aspect of the present invention. However, at least one protease, especially trypsin, FXa, or elastase may be used preferably as the protease to be inhibited.

In the protease inhibition process of the present invention, a protease and the novel polypeptide of the first aspect of the present invention are allowed to react with each other under appropriate reaction conditions such as temperature, pH, time and the like, thereby effecting inhibition of the protease activity. The reaction may be carried out in a test tube or the like (*in vitro*) or in the body of an animal (*in vivo*, *ex vivo*). If necessary, the reaction may be carried out in the presence of other substances, drugs and the like in addition to the novel polypeptide of the first aspect of the present invention. The novel polypeptide to be applied to the enzyme inhibition process of the present invention may be used in the form of a composition containing auxiliary components and the like.

EXAMPLES

Examples of the present invention are given below by way of illustration and not by way of limitation. Abbreviations used herein are based on idiomatical expressions.

Experiments were carried out in the light of the following reports and books.

1. *Labo Manual Genetic Engineering*; M. Muramatsu, 1989, Maruzen
2. *Gene Manipulation Techniques*; Y. Takagi, 1980, Kodansha
3. *Gene Manipulation Manual*; Y. Takagi, 1980, Kodansha
4. *Molecular Cloning, A Laboratory Manual*; T. Maniatis et al., 1982, Cold Spring Harbor Laboratory
5. *Methods in Enzymology*; vol.65, L. Grossman, 1980, Academic Press
6. *Methods in Enzymology*; vol.68, R. Wu, 1979, Academic Press
7. *PCR Protocols, A Guide to Methods and Applications*; Michadel,A.I. et al., 1990, Academic Press
8. *Molecular Cloning, A Laboratory Manual* (second edition); T. Maniatis et al., 1989, Cold Spring Harbor Laboratory

Example 1 Construction of plasmid pM469

Plasmid pM469 to be used later in Example 2 as an expression vector was prepared from plasmid pM463 in the following method. Plasmid pM463 used herein as the starting material is a derivative of plasmid pBR322, which contains a nucleotide sequence for the replication in *E. coli* cells, an ampicillin resistance gene, a tryptophan promoter, a nucleotide sequence coding for an alkaline phosphatase signal peptide and a nucleotide sequence encoding a human pancreatic secretion trypsin inhibitor (to be referred to as "PSTI" hereinafter) (Kanamori,T. *et al.*, *Gene*, vol.66, pp.295 - 300, 1988).

Firstly, plasmid pM463 was double-digested with restriction endonucleases *Hind*III and *Nru*I and the resulting mixture of DNA fragments was applied to 0.7% agarose gel electrophoresis. A DNA fragment of about 3.4 kb thus isolated by the electrophoresis was absorbed to diethylaminoethyl cellulose paper (to be

referred to as "DEAE cellulose paper" hereinafter) and then extracted with a high concentration salt solution (2 M NaCl/10 mM Tris - HCl buffer (pH 7.5)/1 mM EDTA) to recover the DNA fragment of about 3.4 kb.

Separately from this, a linker consisting of an SD sequence, a nucleotide sequence encoding an *E. coli* alkaline phosphatase signal peptide and a nucleotide sequence encoding a part of N-terminal side amino acid sequence of PSTI was divided into six designed fragments. Each of the thus designed fragments was synthesized using a chemical synthesizer (381A, Applied Biosystems, Inc.) (cf. Fig. 1). Of these 6 fragments thus synthesized, S34, S35, S18 and S19 were subjected to 5'-end phosphorylation with T4 polynucleotide kinase (Takara Shuzo Co., Ltd.) in the presence of ATP. Subsequently, each of the combinations of S33 with phosphorylated S34, phosphorylated S35 with phosphorylated S18 and phosphorylated S19 with S20 was subjected to annealing, followed by their ligation making use of a DNA ligation kit (Takara Shuzo Co., Ltd.). The thus ligated sample was then applied to 8% polyacrylamide gel electrophoresis to separate and extract a DNA fragment (linker 1) of about 100 bp which was subsequently purified by phenol treatment and ethanol precipitation. The thus obtained DNA fragment of about 100 bp and the aforementioned DNA fragment of about 3.4 kb were ligated, and *E. coli* HB101 was transformed with the thus ligated product to isolate an ampicillin-resistant colony as a transformant of interest. Plasmid DNA was prepared from the thus obtained transformant and named plasmid pM468 (cf. Fig. 2).

The thus obtained plasmid pM468 was double-digested with *Eco*RI and *Dra*I, and a DNA fragment of about 2.4 kb was isolated and recovered using DEAE cellulose paper in the same manner as described above. Separately from this, plasmid pKC7 containing a kanamycin resistance gene (km^r) (Ngarajarao,R. *et al.*, *Gene*, vol.7, pp.79 - 82, 1979) was double-digested with restriction endonucleases *Eco*RI and *Sma*I to prepare a DNA fragment of about 1.4 kb containing the kanamycin resistance gene. The thus obtained DNA fragment of about 1.4 kb and the aforementioned DNA fragment of about 2.4 kb were ligated, and *E. coli* HB101 was transformed with the thus ligated product to isolate a kanamycin-resistant colony as a transformant of interest. Plasmid DNA was prepared from the thus obtained transformant and named plasmid pM469 (cf. Fig. 3).

Example 2 Production of polypeptide Y46E

A polypeptide, Y46E, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Glu for the 42 position Tyr of the formula 1 sequence counting from its N-terminus was prepared in the following manner.

(1) Cloning of DNA fragment

A DNA fragment encoding the polypeptide Y46E was prepared by means of site-directed mutagenesis (cf. Kunkel,T.A. *et al.*, *Methods in Enzymology*, vol.154, p.367, 1987) using plasmid pM552 (cf. Japanese Patent Application No. 3-325220). The pM552 is a plasmid which has been constructed by inserting into plasmid pM469 a nucleotide sequence that encodes a polypeptide TN70 represented by the following amino acid sequence of formula 3, that is, a DNA fragment (TN70 DNA) represented by the following nucleotide sequence of formula 4. The plasmid pM552 contains a tryptophan promoter, a nucleotide sequence coding for an alkaline phosphatase signal peptide and a kanamycin resistance gene (cf. Fig. 4).

Formula 3

Thr Val Ala Ala Cys Asn Leu Pro Ile Val
Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu
Trp Ala Phe Asp Ala Val Lys Gly Lys Cys
Val Leu Phe Pro Tyr Gly Gly Cys Gln Gly
Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu
Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp
Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn

Formula 4

ACC GTC GCC GCC TGC AAT CTC CCC ATA GTC
CGG GGC CCC TGC CGA GCC TTC ATC CAG CTC
TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC

GTC CTC TTC CCC TAC GGG GGC TGC CAG GGC
AAC GGG AAC AAG TTC TAC TCA GAG AAG GAG
TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT
GGT GAT GAG GAG CTG CTG CGC TTC TCC AAC

Firstly, plasmid pM552 was double-digested with *Hind*III and *Bam*HI, and the thus digested fragments were subjected to 1% agarose gel electrophoresis. A DNA fragment of about 310 bp containing the nucleotide sequence of formula 4 was extracted from the agarose gel and then purified by means of phenol treatment and ethanol precipitation. Using T4 DNA ligase (already described in the foregoing), the thus obtained DNA fragment of about 310 bp was ligated with a phage vector M13mp18 which has been double-digested with *Hind*III and *Bam*HI in advance. Thereafter, the thus ligated product was transfected into *E. coli* JM109 cells (transfection) to obtain M13 phage.

Next, site-directed mutagenesis was carried out making use of a Mutan™ -K kit (Takara Shuzo Co., Ltd.) and according to the manufacturer's instructions. That is, an *E. coli* strain BW313 (HfrKL16PO/45 [*lysA*(61-62)], *dut*1, *ung*1, *thi*-1, *rel*A1) attached to the kit as an indicator strain was infected with the M13 phage to obtain phage plaques. A single plaque was inoculated into 2 x YT medium using the BW313 as an indicator strain and cultured at 37°C for 6 hours. Thereafter, ssDNA (single stranded DNA) was extracted and purified from the resulting phage culture broth by means of phenol treatment and ethanol precipitation. Separately from this, Y46E primer (Fig. 5) for mutagenesis use was synthesized using the aforementioned chemical synthesizer and purified using an OPC column (Applied Biosystems, Inc.), and the 5'-end of the thus purified oligomer Y46E was phosphorylated using T4 polynucleotide kinase and ATP. The thus phosphorylated oligomer was subjected to annealing with the just obtained ssDNA by incubating them at 65°C for 15 minutes and then at 37°C for 15 minutes. The resulting reaction mixture was then incubated at 25°C for 2 hours in the presence of *E. coli* DNA ligase and T4 DNA polymerase attached to the Mutan™ -K kit to synthesize a complementary chain. After terminating the reaction with EDTA, the

resulting complementary chain was transfected into *E. coli* BMH 71 - 18 (*mutS* (Δ(*lac* - *proAB*), *thi*, *supE*, *mutS*215::Tn10(*tet*')/F'*traD*36, *proAB* *lacI*^{q}, *lacZ*ΔM15)) attached to the kit, and phage plaques wer obtained using JM109 as an indicator strain. Phage particles were recovered from each single plaque thus obtained and cultured using JM109 as an indicator strain to obtain a phage solution from which ssDNA was extracted and purified by means of phenol treatment and ethanol precipitation. Thereafter, nucleotide sequence of each ssDNA sample was determined using a DNA sequencer (370A, Applied Biosystems, Inc.) to select an ssDNA sample into which desired mutation has been introduced.

Next, PCR was carried out in the following method, using the thus obtained ssDNA as a template. That is. a *Sca*I primer (Fig. 6) to be used as a sense primer and a *Bam*HI primer (Fig. 7) to be used as an antisense primer were synthesized using the aforementioned chemical synthesizer and then purified using the aforementioned OPC column. The thus obtained *Sca*I sense primer and *Bam*HI antisense primer were added to a 100 µl solution containing the aforementioned ssDNA, and the resulting mixture was subjected to the PCR reaction using Gene Amp^R PCR Reagent Kit (Takara Shuzo Co., Ltd.) by repeating 30 cycles of the reaction. In this instance, each reaction cycle was effected by a series of incubation at 94°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes in that order. Thereafter, the DNA fragment of the present invention was purified from the resulting reaction mixture by means of phenol treatment and ethanol precipitation.

(2) Construction of expression vector

Plasmid pM469 obtained in Example 1 was double-digested with *Sca*I and *Bam*HI, and the resulting DNA fragments were subjected to 0.7% low melting agarose gel electrophoresis. A DNA fragment of about 3.5 kb was purified from the agarose gel by cutting out a gel portion containing the fragment, melting the cut out gel portion at 65°C and then treating the resulting solution with phenol, followed by ethanol precipitation. Next, the DNA fragment of the present invention obtained in the above procedure (1) was digested with *Bam*HI and then ligated with the just obtained DNA fragment of about 3.5 kb to obtain an expression plasmid pM575B (cf. Fig. 8).

The thus obtained plasmid pM575B was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 310 bp was extracted and purified by means of phenol treatment and ethanol precipitation. The thus purified DNA fragment was ligated with each of the phage vectors M13mp18 and M13mp19 (Takara Shuzo Co., Ltd.) which have been double-digested with *Hind*III and *Bam*HI in advance, and each of the ligated products was transfected into *E. coli* JM109 to form plaques. Thereafter, ssDNA was prepared from each of the resulting plaques and subjected to sequencing using a DNA sequencer (370A, Applied Biosystems, Inc.). The thus confirmed nucleotide sequence of a region of plasmid pM575B from its *Hind*III site to *Bam*HI site containing the DNA fragment of interest and its corresponding amino acid sequence are shown in Fig. 9 (see Sequence ID No. 1 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM575B), was prepared by transforming *E. coli* JE5505 with the plasmid pM575B obtained in the above procedure (2) in accordance with the Hanahan's method (Hanahan D., Techniques for Transformation of *E. coli*; in *DNA Cloning*, vol.1, Glover, D.M. (ed.), pp.109 - 136, IRL Press, 1985). The thus prepared transformant, *E. coli* JE5505 (pM575B), was cultured at 37°C for about 8 hours in L-broth containing 50 µg/ml of kanamycin (first seed culture). The resulting culture broth was inoculated in about 100 volumes of the same medium and cultured overnight at 37°C (second seed culture). The main culture was then carried out by inoculating the resulting culture broth in 50 volumes of M9CA medium containing 50 µg/ml of kanamycin and culturing at 37°C for about 1 hour. After adding 3-β-indole acrylic acid (Wako Pure Chemical Industries, Ltd.) to a final concentration of 10 µg/ml, the culturing was continued for additional 16 hours. Thereafter, culture filtrate was obtained from the resulting culture broth by centrifugation.

The culture filtrate thus prepared was diluted with 0.1% BSA (bovine serum albumin)/0.2 M triethanolamine-HCl buffer (pH 7.8) to each predetermined concentration, and the diluted samples were used for the measurement of trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture filtrate of a transformant, *E. coli* JE5505 (pM553), was used as a control in the activity measurement, which has been obtained by deleting the aforementioned nucleotide sequence of formula 4 from plasmid pM552 to obtain a plasmid pM553 and by transforming *E. coli* JE5505 with the thus obtained plasmid pM553.

As the results, markedly high trypsin-inhibiting activity was found in the culture filtrate of *E. coli* JE5505 (pM575B) in comparison with the control. The transformant *E. coli* JE5505 (pM575B) has been deposited by the present inventors on July 16, 1991, in Ferm ntation Research Institut , Agency of Industrial Science and Technology, and has been assigned the designation as FERM P-12357 which was subsequently transferred to the International Depositary Authority on October 17, 1991, as FERM BP-3613.

(4) Purification of inventive polypeptide from culture supernatant of *E. coli* JE5505 (pM575B)

The novel polypeptide of the present invention was recovered and purified in the following method from culture supernatant of the transformant *E. coli* JE5505 (pM575B) obtained in the above procedure (3).

(a) Ammonium Sulfate Precipitation

Ammonium sulfate was added to one liter of the culture supernatant to a level of 80% saturation. The mixture was stirred until ammonium sulfate was completely dissolved and then the solution was allowed to stand overnight at 4°C. The sample was cetrifuged at 12,000 x g for 30 minutes at 4°C, and the thus obtained pellet was dissolved in 25 ml of distilled water. After removing insoluble precipitation by centrifugation, the supernatant was concentrated to 1 ml using an ultrafiltration membrane (molecular weight cutoff of 1,000; Diaflow membrane YM-1, Grace Company). Thereafter, the thus concentrated sample was centrifuged at 5,860 x g for 10 minutes at 4°C to recover supernatant.

(b) Gel filtration

The concentrated sample obtained in the above step (a) was applied to a column (1 cmØ x 115 cm) packed with Sephadex G-50 (Pharmacia) which has been equilibrated with PBS^- (phosphate buffered saline). The loaded sample was eluted by PBS^- at a flow rate of 0.2 ml/min to obtain 2 ml fractions.

A portion of each fraction was collected and checked for its trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1). Thereafter, active fractions were pooled and dialyzed overnight at 4°C against 20 mM Tris-HCl buffer (pH 8.5) using a dialysis membrane (molecular weight cutoff of 1,000; Spectrum Medical Industries, Inc.).

(c) Anion exchange chromatography

Anion exchange chromatography was carried out using an FPLC system (Pharmacia) in the following method. The dialyzed sample obtained in the above step (b) was applied to Mono Q column (5 mmØ x 50 mm, Pharmacia) which has been equilibrated with 20 mM Tris-HCl (pH 8.5). Elution was carried out at a flow rate of 1 ml/min with a linear density gradient of 0 to 0.4 M NaCl/20 mM Tris-HCl (pH 8.5)/48 min. Protein concentration in the eluent was monitored by absorbance at 280 nm and each protein peak was fractioned. A portion of each collected fraction was checked for its trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1) and active fractions were pooled.

(d) Reverse phase chromatography

The active fraction pooled in the above step (c) was applied to Vydac C18 column (4.6 mmØ x 25.0 cm, The Separations Group) which has been equilibrated with 0.04% trifluoroacetic acid solution. Using Waters 625 LC system (Waters Associates, Inc.), elution was carried out at a flow rate of 1 ml/min with a linear density gradient of 0 to 100% acetonitrile/0.04% trifluoroacetic acid/30 min. Protein concentration in the eluent was monitored by absorbance at 280 nm and each protein peak was fractioned. A portion of each fraction was checked for its trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1) and active fractions were pooled. The pooled sample was dried under a reduced pressure using a centrifugation vacuum concentrator (Tomy Seiko Co., Ltd.) to obtain a purified polypeptide sample. The purified polypeptide was used for the SDS-polyacrylamide gel electrophoresis in the following step (5), the amino acid sequence analysis in the step (6) and the activity measurement in Example 12.

(5) SDS-polyacrylamide gel electrophoresis

The purified polypeptide sample obtained in the above procedure (4) was subjected to SDS-polyacrylamide gel electrophoresis (to be referred to as "SDS-PAGE" hereinafter) in accordance with

Laemmli's method (Laemmli,U.K., *Nature*, vol.227, pp.680 - 685, 1970) That is, the purified polypeptide was dissolved in 100 µl of distilled water, and a portion of the solution was mixed with the same volume of Seprasol II (Daiichi Pure Chemicals Co., Ltd.). The mixture was treated at 100°C for 5 minutes and th n electrophoresed with 15% gel (8 cm x 9 cm, 1 mm in thickness) at 15 mA for 50 minutes and then at 30 mA for 35 minutes. In this instance, a commercial molecular weight marker kit was used (Electrophoresis Calibration Kit, Pharmacia). After the electrophoresis, staining was carried out with a commercial silver staining kit (2D - Silver Staining Reagent II, Daiichi Pure Chemicals Co., Ltd.). The purified sample showed a single band by SDS - PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined using Model 477A Protein Sequencing System 120A PTH Analyzer (Applied Biosystems Inc.). Identification of the amino acid sequence was determined by detecting PTH - amino acid at an absorbance of 270 nm, based on the retention time of standard PTH - amino acids (Applied Biosystems Inc.) which have been isolated in the same procedure.

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (4) was the polypeptide of interest Y46E (cf. Sequence ID No. 2 in SEQUENCE LISTING).

Example 3 Production of polypeptide Q19K

A polypeptide, Q19K, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Lys for the 15 position Gln of the formula 1 sequence counting from its N - terminus was prepared in the following manner.

(1) Cloning of DNA fragment

Plasmid pM558 was derived from the aforementioned plasmid pM552 in the following manner. Firstly, an oligomer TV12DD (Fig. 10) for mutation introduction use was synthesized chemically. Next, ssDNA was prepared using M13 phage obtained in Example 2 (1) from *E. coli* JM109, in the same manner as in Example 2 (1) making use of the aforementioned Mutan™ - K kit, and the desired mutation was introduced into the ssDNA using the TV12DD. Thereafter, PCR was carried out in accordance with the procedure described in Example 2 (1) using the thus mutation - introduced ssDNA as a template. In this instance, *Sca*I sense primer prepared in Example 2 (1) was used as the sense primer and an M13 primer RV (Takara Shuzo Co., Ltd.) was used as the antisense primer. The thus obtained PCR product was digested with *Bam*HI and inserted into plasmid pM469 in the same manner as in Example 2 (2) to obtain plasmid pM558.

Site - directed mutagenesis was carried out by PCR, the method of Landt *et al.* (Landt, O. *et al.*, *Gene*, vol.96, pp.125 - 128, 1990), using the thus obtained plasmid pM558 as a template for PCR use. Firstly, a *Hind*III primer (Fig. 11) and a Q19K primer (Fig. 12) to be used respectively as a sense primer and an antisense primer in the first PCR were synthesized chemically. First PCR was carried out making use of the aforementioned Gene Amp^R PCR Reagent Kit, using the thus synthesized sense and antisense primers and plasmid pM558 as a template and repeating 30 cycles of the reaction. In this instance, each reaction cycle was effected by a series of incubation at 94°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes in that order. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 160 bp. The DNA fragment of interest was then extracted and purified from the gel by means of phenol treatment and ethanol precipitation, and the thus purified DNA fragment was dissolved in TE buffer. Thereafter, second PCR was carried out in the same manner as the above first PCR using the thus dissolved DNA fragment as a sense primer and the plasmid pM558 as a template. In this instance, pBR *Bam*HI primer (Fig. 13) prepared in Example 2 - (2) obtained by chemical synthesis was used as an antisense primer.

A portion of the amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 350 bp. The DNA fragment was then extracted and purified from the gel by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

(2) Construction of expression vector

The plasmid pM463 (Kanamori T. *et al.*) described in the foregoing was double-digested with *Hind*III and *Bam*HI, and the thus digested mixture was subjected to 0.7% low melting point agarose gel electrophoresis to obtain a DNA fragment of about 3.3 kb. Next, the DNA fragment of the present invention having a size of about 350 bp obtained in the above step (1) was double-digested with *Hind*III and *Bam*HI, and the thus digested fragment was ligated with the just described DNA fragment of about 3.3 kb using the aforementioned T4 DNA ligase, thereby obtaining an expression plasmid pM576 (cf. Figs. 14 and 15).

The thus obtained expression plasmid pM576 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 310 bp was extracted and purified. The thus purified DNA fragment of interest was ligated with each of the aforementioned phage vectors M13mp18 and M13mp19 which have been double-digested with *Hind*III and *Bam*HI in advance, and the thus ligated product was transfected into *E. coli* JM109. Thereafter, ssDNA was prepared from each of the resulting plaques and subjected to sequencing using the aforementioned DNA sequencer. Thus confirmed nucleotide sequence of a region of plasmid pM576 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 16 (cf. Sequence ID No. 3 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM576), was isolated by transforming *E. coli* JE5505 with the plasmid pM576 obtained in the above procedure (2), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 2 (3) to recover culture supernatant, except that ampicillin was added to the culture medium to a final concentration of 50 μg/ml in stead of kanamycin.

The culture supernatant was diluted with 0.1% BSA/0.2 M triethanolamine-HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant of a transformant, *E. coli* JE5505 (pM463C), was used as a control in the activity measurement, which has been obtained by deleting the polypeptide Q19K-encoding nucleotide sequence from plasmid pM576.

As the results, markedly high trypsin-inhibiting activity was found in the culture supernatant of *E. coli* JE5505 (pM576) in comparison with the control. The transformant *E. coli* JE5505 (pM576) has been deposited by the present inventors on July 16, 1991, in Fermentation Research Institute, Agency of Industrial Science and Technology, and has been assigned the designation as FERM P-12358 which was subsequently transferred to the International Depositary Authority on October 17, 1991, as FERM BP-3614.

(4) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM576)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (3) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in accordance with the procedure described in Example 2 (4). The obtained purified sample was used for the following SDS-PAGE (5), amino acid analysis (6) and activity measurement in Example 12.

(5) SDS-PAGE

The purified polypeptide sample obtained in the above procedure (4) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (4) was the polypeptide Q19K of the present invention (cf. Sequence ID No. 4 of the SEQUENCE LISTING).

Example 4 Production of polypeptide Q19R

A polypeptide, Q19R, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Arg for the 15 position Gln of the formula 1 sequence counting from its N-terminus was prepared in the following method.

(1) Cloning of DNA fragment

Plasmid pM594 was derived from the aforementioned plasmid pM552 in the following procedure. Firstly, an AN68 primer (Fig. 17) was synthesized chemically. Using the aforementioned pM552 as a template, the thus synthesized AN68 primer as an antisense primer and the *Hind*III primer prepared in Example 3 (1) as a sense primer, first PCR was carried out in accordance with the procedure described in Example 3 (1) making use of the aforementioned Gene Amp^R PCR Reagent Kit. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 130 bp. The DNA fragment of interest was then extracted and purified from the gel by means of phenol treatment and ethanol precipitation, and the thus purified DNA fragment was dissolved in TE buffer. Thereafter, second PCR was carried out in the same manner as the above first PCR using the thus dissolved DNA fragment as a sense primer and the plasmid pM552 as a template. In this instance, pBR *Bam*HI primer prepared in Example 3 (1) was used as an antisense primer. A portion of the amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 350 bp. The DNA fragment was extracted and purified from the gel by means of phenol treatment and ethanol precipitation, and the thus purified fragment was digested with *Hind*III and *Bam*HI to obtain a DNA fragment of about 300 bp. Thereafter, the thus obtained DNA fragment was inserted into plasmid pM463 in the same manner as in Example 3 (2) to obtain plasmid pM594.

Next, site-directed mutagenesis was carried out by the method of Landt *et al.* in the following manner. Firstly, a *Sac*II primer (Fig. 18) and a Q19R primer (Fig. 19) were obtained by chemical synthesis to be used respectively as a sense primer and an antisense primer. First PCR was carried out in the same manner as described above, using the thus synthesized sense and antisense primers and the aforementioned plasmid pM594 as a template. A portion of the amplified product by the first PCR was applied to 4% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 70 bp. The DNA fragment of interest was then extracted and purified from the gel by means of phenol treatment and ethanol precipitation, and the thus purified DNA fragment was dissolved in TE buffer. Thereafter, second PCR was carried out in the same manner as the above first PCR using the thus dissolved DNA fragment as a sense primer and the plasmid pM594 as a template. In this instance, pBR *Bam*HI primer prepared in Example 3 (1) was used as an antisense primer. A portion of the amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 260 bp. The DNA fragment was then extracted and purified from the gel by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

(2) Construction of expression vector

The plasmid pM594 was double-digested with *Sac*II and *Bam*HI, and a DNA fragment of interest having a size of about 3.4 kb was extracted and purified from the digested sample. The thus purified DNA fragment was ligated with the DNA fragment of about 260 bp obtained in the above step (1) using the aforementioned T4 DNA ligase, thereby obtaining an *E. coli* expression plasmid pM735 (cf. Figs. 20 and 21). The thus obtained plasmid pM735 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 300 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer.

Thus confirmed nucleotide sequence of a region of plasmid pM735 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 22 (cf. Sequence ID No. 5 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM735), was isolated by transforming *E. coli* JE5505 with the plasmid pM735 obtained in the above procedure (2), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover culture supernatant.

The culture supernatant thus prepared was diluted with 0.1% BSA/0.2 M triethanolamine – HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin – inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant of the transformant, *E. coli* JE5505 (pM463C), prepared in Example 3 (3) was used as a control in the activity measurement. As the results, markedly high trypsin – inhibiting activity was found in the culture filtrate of *E. coli* JE5505 (pM735) in comparison with the control.

The transformant *E. coli* JE5505 (pM735) has been deposited by the present inventors on May 1, 1992, in Fermentation Research Institute, Agency of Industrial Science and Technology, and has been assigned the designation as FERM P – 12945.

(4) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM735)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (3) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in accordance with the procedure described in Example 2 (4). The thus obtained purified sample was used for the following SDS – PAGE (5), amino acid analysis (6) and activity measurement in Example 17.

(5) SDS – PAGE

The purified polypeptide sample obtained in the above procedure (4) was subjected to SDS – PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS – PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (4) is the polypeptide Q19R of the present invention (cf. Sequence ID No. 6 of the SEQUENCE LISTING).

Example 5 Production of polypeptide Q19K/Y46E

A polypeptide, Q19K/Y46E, having an, amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Lys for the 15 position Gln, as well as Glu for the 42 position Tyr, of the formula 1 sequence counting from its N – terminus was prepared in the following manner.

(1) Cloning of DNA fragment

First PCR was carried out in the same manner as in Example 3 (1), making use of the aforementioned Gene Amp^R PCR Reagent Kit and using the *Hind*III primer and Q19K primer prepared in Example 3 (1) as sense and antisense primers and the plasmid pM594 prepared in Example 4 (1) as a template. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 170 bp. The DNA fragment of interest was then extracted and purified from the gel by means of phenol treatment and ethanol precipitation, and the thus purified DNA fragment was dissolved in TE buffer. Thereafter, second PCR was carried out in the same manner as the above first PCR using the thus dissolved DNA fragment as a sense primer and plasmid pM575B as a template. In this instance, pBR *Bam*HI primer prepared in Example 3 (1) was used as an antisense primer. A portion of the amplified product by the second PCR was applied to 1.5% agarose gel

electrophoresis to confirm formation of a DNA fragment of interest having a size of about 350 bp. The DNA fragment was then extracted and purified by m ans of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

(2) Construction of expression vector

The DNA fragment of the present invention obtained in the above step (1) was inserted into plasmid pM463 in accordance with the procedure of Example 3 (2) to prepare an expression plasmid pM736 (cf. Fig. 23). The thus prepared plasmid pM736 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 300 bp was extracted and purified. The thus purified DNA fragment of interest was subjected to sequencing using the aforementioned DNA sequencer in the same manner as described in Example 2 (2). Thus confirmed nucleotide sequence of a region of plasmid pM736 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 24 (cf. Sequence ID No. 7 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM736), was isolated by transforming *E. coli* JE5505 with the plasmid pM736 obtained in the above procedure (2). The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover culture supernatant.

The culture supernatant thus prepared was diluted with 0.1% BSA/0.2 M triethanolamine-HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant of the transformant, *E. coli* JE5505 (pM463C), prepared in Example 3 (3) was used as a control in the activity measurement. As the results, markedly high trypsin-inhibiting activity was found in the culture supernatant of *E. coli* JE5505 (pM736) in comparison with the control.

The transformant *E. coli* JE5505 (pM736) has been deposited by the present inventors on May 1, 1992, in Fermentation Research Institute, Agency of Industrial Science and Technology, and has been assigned the designation as FERM P-12946.

(4) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM736)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (3) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in accordance with the procedure described in Example 2 (4). The thus purified polypeptide sample was used for the following SDS-PAGE (5), amino acid analysis (6) and activity measurement in Example 17.

(5) SDS-PAGE

The purified polypeptide sample obtained in the above procedure (4) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (4) is the polypeptide Q19K/Y46E of the present invention (cf. Sequence ID No. 8 of the SEQUENCE LISTING).

Example 6 Production of polypeptide R11E/Y46E

A polypeptide, R11E/Y46E, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Glu for the 7 position Arg, as well as Glu for the 42 position Tyr, of the formula 1 sequence counting from its N-terminus was prepared in the following method.

(1) Cloning of DNA fragment

An R11E primer (Fig. 25) was synthesized chemically to be used as an antisense primer. Using the antisense primer thus synthesized and the *Hind*III sense primer prepared in Example 3 (1), first PCR was carried out in accordance with the procedure described in Example 3 (1), making use of the aforementioned Gene Amp^R PCR Reagent Kit. In this instance, plasmid pM594 prepared in Example 4 (1) was used as a template. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm presence of a band of interest having a size of about 150 bp. The thus amplified product was extracted and purified by means of phenol treatment and ethanol precipitation, and the purified product was dissolved in TE buffer. Thereafter, second PCR was carried out in the same manner as the above first PCR using the thus dissolved product as a sense primer and the aforementioned plasmid pM575B as a template. In this instance, pBR *Bam*HI primer prepared in Example 3 (1) was used as an antisense primer. A portion of the amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 350 bp. The DNA fragment was then extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

(2) Construction of expression vector

The DNA fragment of the present invention having a size of about 350 bp prepared in the above step (1) was inserted into plasmid pM463 in the same manner as in Example 3 (2) to obtain an expression plasmid pM726B (cf. Fig. 26). The thus obtained expression plasmid pM726B was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 300 bp was extracted and purified. The thus purified DNA fragment of interest was subjected to sequencing using the aforementioned DNA sequencer in the same manner as in Example 2 (2).

Thus confirmed nucleotide sequence of a region of plasmid pM726B from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 27 (cf. Sequence ID No. 9 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM726B), was isolated by transforming *E. coli* JE5505 with the plasmid pM726B obtained in the above procedure (2), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover culture supernatant.

The culture supernatant thus prepared was diluted with 0.1% BSA/0.2 M triethanolamine-HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin-inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant of the transformant, *E. coli* JE5505 (pM463C), obtained in Example 3 (3) was used as a control in the activity measurement. As the results, markedly high trypsin-inhibiting activity was found in the culture supernatant of *E. coli* JE5505 (pM726B) in comparison with the control.

(4) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM726B)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (3) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in accordance with the procedure described in Example 2 (4). The purified polypeptide sample was used for the following SDS-PAGE (5), amino acid analysis (6) and activity measurement in Example 17.

(5) SDS - PAGE

The purifi d polypeptide sample obtained in the above procedure (4) was subjected to SDS - PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS - PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (4) is the aimed polypeptide R11E/Y46E of the present invention (cf. Sequence ID No. 10 of the SEQUENCE LISTING).

Example 7 Production of polypeptide Y46E - AN

A polypeptide, Y46E - AN, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Glu for the 42 position Tyr of the formula 1 sequence counting from its N - terminus was prepared in the following manner.

(1) Cloning of DNA fragment and construction of expression vector

PCR was carried out in the same manner as in Example 3 (1), making use of the aforementioned Gene Amp^R PCR Reagent Kit and using the *Sac*II primer prepared in Example 4 (1) and the pBR *Bam*HI primer prepared in Example 3 (1) as sense and antisense primers and the aforementioned plasmid pM575B as a template. A portion of the amplified product by the PCR was applied to 1.5% agarose gel electrophoresis to confirm presence of a single band of interest having a size of about 250 bp. The thus amplified product was extracted and purified by means of phenol treatment and ethanol precipitation, and the thus purified product was digested with *Sac*II and *Bam*HI to obtain a DNA fragment of about 220 bp. The thus obtained DNA fragment of about 220 bp was ligated with a DNA fragment having a size of about 3.4 kb which have been prepared from plasmid pM594 by double - digesting it with *Sac*II and *Bam*HI, thereby obtaining an expression plasmid pM575C (cf. Fig. 28). The thus prepared plasmid pM575C was double - digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 300 bp was extracted and purified. Thereafter, the thus purified DNA fragment was subjected to sequencing using the aforementioned DNA sequencer in the same manner as described in Example 2 (2).

Thus confirmed nucleotide sequence of a region of plasmid pM575C from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 29 (cf. Sequence ID No. 11 in the SEQUENCE LISTING).

(2) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM575C), was isolated by transforming *E. coli* JE5505 with the plasmid pM575C obtained in the above procedure (1), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover culture supernatant.

The culture supernatant thus prepared was diluted with 0.1% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin - inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant of the transformant, *E. coli* JE5505 (pM463C), obtained in Example 3 (3) was used as a control in the activity measurement. As the results, markedly high trypsin - inhibiting activity was found in the culture supernatant of *E. coli* JE5505 (pM575C) in comparison with the control. (3) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM575C)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (2) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in accordance with the procedure described in Example 2 (4). The thus obtained purified sample was used for the following SDS - PAGE (4), amino acid

analysis (5) and activity measurement in Example 17.

(4) SDS - PAGE

The purified polypeptide sample obtained in the above procedure (3) was subjected to SDS - PAGE in accordance with the procedure described in Example 2 (5). After completion of the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS - PAGE.

(5) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (3) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (3) is the aimed polypeptide Y46E - AN of the present invention (cf. Sequence ID No. 12 of the SEQUENCE LISTING).

Example 8 Production of polypeptide Q19K - AN

A polypeptide, Q19K - AN, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Lys for the 15 position Gln of the formula 1 sequence counting from its N - terminus was prepared in the following method.

(1) Cloning of DNA fragment and construction of expression vector

First PCR was carried out in the same manner as in Example 3 (1), making use of the aforementioned Gene Amp^R PCR Reagent Kit and using the *Sac*II primer prepared in Example 4 (1) and the pBR *Bam*HI primer prepared in Example 3 (1) as sense and antisense primers and the aforementioned plasmid pM576 as a template. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm presence of a single band of interest having a size of about 250 bp. The thus amplified product was extracted and purified by means of phenol treatment and ethanol precipitation, and the thus purified product was digested with *Sac*II and *Bam*HI to obtain a DNA fragment of about 220 bp. The thus obtained DNA fragment of about 220 bp was ligated with a DNA fragment having a size of about 3.4 kb which have been prepared from plasmid pM594 by double - digesting it with *Sac*II and *Bam*HI, thereby obtaining an expression plasmid pM576B (cf. Fig. 30). The thus prepared plasmid pM576B was double - digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 300 bp was extracted and purified. Thereafter, the thus purified DNA fragment was subjected to sequencing using the aforementioned DNA sequencer in the same manner as described in Example 2 (2).

Thus confirmed nucleotide sequence of a region of plasmid pM576B from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 31 (cf. Sequence ID No. 13 in the SEQUENCE LISTING).

(2) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM576B), was isolated by transforming *E. coli* JE5505 with the plasmid pM576B obtained in the above procedure (1), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover culture supernatant.

The culture supernatant thus prepared was diluted with 0.1% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin - inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant of the transformant, *E. coli* JE5505 (pM463C), obtained in Example 3 (3) was used as a control in the activity measurement. As the results, markedly high trypsin - inhibiting activity was found in the culture supernatant of *E. coli* JE5505 (pM576B) in comparison with the control.

(3) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM576B)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (2) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in that order in accordance with the procedure described in Example 2 (4). The thus obtained purified sample was used for the following SDS - PAGE (4), amino acid analysis (5) and activity measurement in Example 17.

(4) SDS - PAGE

The purified polypeptide sample obtained in the above procedure (3) was subjected to SDS - PAGE in accordance with the procedure described in Example 2 (5). After completion of the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS - PAGE.

(5) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (3) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (3) is the polypeptide Q19K - AN of the present invention (cf. Sequence ID No. 14 of the SEQUENCE LISTING).

Example 9 Production of polypeptide Q19R/Y46E

A polypeptide, Q19R/Y46E, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Arg for the 15 position Gln, as well as Glu for the 42 position Tyr, of the formula 1 sequence counting from its N - terminus was prepared in the following manner.

(1) Cloning of DNA fragment

First PCR was carried out in accordance with the procedure described in Example 3 (1), making use of the aforementioned Gene Amp^R PCR Reagent Kit and using the *Hind*III primer prepared in Example 3 (1) and the Q19R primer prepared in Example 4 (1) as sense and antisense primers and the plasmid pM594 prepared in Example 4 (1) as a template. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm presence of a band of interest having a size of about 170 bp. The thus amplified product was extracted and purified by means of phenol treatment and ethanol precipitation, and the thus purified DNA fragment was dissolved in TE buffer. Thereafter, second PCR was carried out in the same manner as the above first PCR using the thus dissolved DNA fragment as a sense primer and the aforementioned plasmid pM575B as a template. In this instance, pBR *Bam*HI primer prepared in Example 3 (1) was used as an antisense primer. A portion of the amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm presence of a band of interest having a size of about 350 bp. The thus amplified product was then extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

(2) Construction of expression vector

The DNA fragment of the present invention having a size of about 350 bp prepared in the above step (1) was inserted into plasmid pM463 in the same manner as in Example 3 (2) to obtain an expression plasmid pM737B (cf. Fig. 32). The thus obtained expression plasmid pM737B was double - digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 300 bp was extracted and purified. The thus purified DNA fragment of interest was subjected to sequencing using the aforementioned DNA sequencer in the same manner as in Example 2 (2).

Thus confirmed nucleotide sequence of a region of plasmid pM737B from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 33 (cf. Sequence ID No. 15 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM737B), was isolated by transforming *E. coli* JE5505 with the plasmid pM737B obtained in the above procedure (2), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover culture supernatant.

The culture supernatant thus prepared was diluted with 0.1% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8), and the diluted samples were used for the measurement of trypsin - inhibiting activity in accordance with the procedure described in Example 17 (1). In this instance, culture supernatant Of a transformant, *E. coli* JE5505 (pM463C), obtained in Example 3 (3) was used as a control in the activity measurement. As the results, markedly high trypsin - inhibiting activity was found in the culture supernatant of *E. coli* JE5505 (pM737B) in comparison with the control.

(4) Purification of the novel polypeptide of the present invention from culture supernatant of *E. coli* JE5505 (pM737B)

The polypeptide of the present invention was purified from the culture supernatant obtained in the above procedure (3) by means of ammonium sulfate precipitation, gel filtration, anion exchange chromatography and reverse phase chromatography in accordance with the procedure described in Example 2 (4). The thus purified polypeptide sample was used for the following SDS - PAGE (5), amino acid analysis (6) and activity measurement in Example 17.

(5) SDS - PAGE

The purified polypeptide sample obtained in the above procedure (4) was subjected to SDS - PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS - PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6).

As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (4) is the polypeptide Q19R/Y46E (cf. Sequence ID No. 16 of the SEQUENCE LISTING).

Example 10 Production of polypeptide Q19K Y46D

A polypeptide, Q19K/Y46D, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Lys for the 15 position Gln, as well as Asp for the 42 position Tyr, of the formula 1 sequence counting from its N - terminus was prepared in the following method.

(1) Construction of plasmid pM748

PCR was carried out twice in accordance with the procedure described in Example 3 (1) using a plasmid pM710 as a template. In this instance, the plasmid pM710 was prepared in the following method. That is, a DNA fragment represented by the nucleotide sequence Liker 710 shown in Fig. 34 was divided and designed into 5 small fragments, and each of the thus designed fragments was synthesized chemically in accordance with the procedure described in Example 1, making use of the aforementioned chemical synthesizer. Separately from this, the plasmid pM594 prepared in Example 4 (1) was double - digested with *Hind*III and *Apa*I to obtain a DNA fragment of about 3.2 kbp. The thus obtained DNA fragment was ligated with the chemically synthesized DNA fragment, making use of the aforementioned T4 DNA ligase, to obtain the expression plasmid pM710.

PCR was carried out in the following method. First PCR was carried out using a chemically synthesized Y46D primer (Fig. 35) as a sense primer and the pBR *Bam*HI primer prepared in Example 3 (1) as an antisense primer. A portion of amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 120 bp. The thus

amplified product was extracted and purifi d by means of phenol treatment and ethanol pr cipitation and then dissolved in TE buffer.

Thereafter, second PCR was carri d out using the thus dissolved DNA fragment as an antisense primer and the *Hind*III primer prepared in Example 3 (1) as a sense primer. Thereby a portion of amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 380bp.

The thus obtained amplified product was then extracted and purified by means of phenol treatment and ethanol precipitation. Then, the DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM748.

(2) Construction of plasmid pM727

PCR was carried out in the same manner as in Example 3 (1) using the thus obtained plasmid pM748 as a template. First PCR was carried out using the *Hind*III primer and the Q19K primer prepared in Example 3 (1) as a sense and antisense primers, respectively. A portion of the amplified product by the first PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 210 bp. The thus amplified product was then extracted and purified by means of phenol treatment and ethanol precipitation and then dissolved in TE buffer. Thereafter, second PCR was carried out using the thus dissolved DNA fragment as a sense primer and the pBRBamHI primer prepared in Example 3 (1) as an antisense primers. Thereby a portion of amplified product by the second PCR was applied to 1.5% agarose gel electrophoresis to confirm formation of a DNA fragment of interest having a size of about 380bp.

The thus amplified product was then extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

The thus obtained DNA fragment of interest having a size of about 380 bp was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM727. The thus constructed plasmid pM727 was double-digested with *Hind*III and *Bam*HI, and a fragment of interest having a size of about 340 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer. The confirmed nucleotide sequence of a region of plasmid pM727 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 36 (cf. Sequence ID No. 17 in the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM727), was isolated by transforming *E. coli* JE5505 with the plasmid pM727 obtained in the above procedure (2), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3). The resulting culture medium was concentrated using an ultrafiltration membrane, Benchmark GX membrane (Menbre Inc. pore size 0.2 μm), thereafter the thus concentrated sample was centrifuged at 10,000 xg for 20 minutes at 4°C to recover the cells.

(4) Solubilization and reduction

The cells obtained in the above procedure (2) were suspended in 0.5% Triton X-100/10 mM EDTA solution and homogenized using high pressure laboratory homogenizer (RANNI a/s.) with 800 bar. The thus treated suspension was centrifuged at 10,000 xg for 20 minutes at 4°C to recover the inclusion body.

The pellet was suspended by 0.5% Triton X-100/10 mM EDTA solution and then it was centrifuged again. After repeating centrifugation twice in the same procedure as above, the pellet was recovered. The thus obtained pellet was resolved by an appropriate amount of a solubilization buffer (5 M guanidine hydrochloride, 0.005% Tween 80, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 2 mM glutathione in reduced form and 0.02 mM glutathione in oxidized form). And then 2-mercaptoethanol was added to the solution to final concentration 50 μM . The prepared solution was stirred overnight at 4°C and thereafter concentrated by ultrafiltration using a membrane, YM-5 (Grace Japan) followed by filtration using a filter of 0.44 μm pore size.

The resulting filtrate was applied to a column (5 cmø x 95 cm) packed with Sephacryl S-100 HR (Pharmacia) which has been equilibrated with the aforementioned solubilization buffer. The loaded sample was then eluted using the same buffer at a flow rate of 3.5 ml/min and each 30 ml fraction was collected.

During the elution, protein concentration was monitored at 280 nm. A portion of each collected fraction was subjected to SDS-PAGE. After staining with Coomassie Brilliant Blue, fractions with a desired molecular weight was pooled.

The pooled fraction was diluted by the aforementioned solubilization buffer to 0.5 mg/ml. The solution was used as a sample for the following refolding treatment.

(5) Refolding

Refolding was carried out by dialyzing the sample obtained in the above procedure (3) under the following conditions. That is, the sample was dialized by two changes of 10 to 15 volumes of solubilization buffer eliminated guanidine hydrochloride, followed by additional two changes of 10 to 15 volumes of distilled water. After the dialysis, the sample was adjusted to pH 2 with HCl and subjected to the following purification process.

(6) Purification

(a) Reverse phase chromatography

The sample obtained in the above procedure (5) was applied to PLRP-S column (25 mmØ x 150 mm, Polymer Laboratories) which has been equilibrated with 0.1% TFA solution. Elution was carried out at a flow rate of 5 ml/min with a linear density gradient of 0-70% acetonitrile/0.1% TFA/30 min, 70-100% acetonitrile/0.1% TFA/3 min. Protein concentration in the eluent was monitored at 280 nm and each 5 ml fraction was collected. A portion of each collected fraction was checked for its trypsin-inhibiting activity in accordance with the procedure of Example 17 (1). Fractions showing the trypsin-inhibiting activity were pooled, lyophilized and then dissolved in 70% formic acid to a final concentration of about 100 μM. The resulting solution was mixed with 2,000 times higher molar ratio of cyanogen bromide and then stood in the dark for 24 hours at 25°C. The thus treated solution was diluted with 2 volumes of distilled water.

(b) Cation exchange chromatography

The sample obtained in the above step (a) was applied to SP-Toyopearl column (30 mmØ x 150 mm, Tosoh Corp.) which has been equilibrated with 10% formic acid solution. Using the FPLC system, elution was carried out at a flow rate of 8 ml/min with a linear density gradient of NaCl (NaCl concentration 0-1.2M/10 min). Protein concentration in the eluent was monitored by measuring absorbance at 280 nm and 32 ml fractions was collected. A portion of each collected fraction was checked for its trypsin-inhibiting activity in accordance with the procedure described in Example 16 (1). A fraction showing the activity was used in the following reverse phase chromatography.

(c) Reverse phase chromatography

The active fraction obtained in the above step (b) was applied to the aforementioned PLRP-S column (25 mmØ x 150 mm) which has been equilibrated with 0.1% TFA solution. Elution was carried out at a flow rate of 10 ml/min with a linear density gradient of 0-70% acetonitrile/0.1% TFA/15 min and 70-100% acetonitrile/0.1% TFA/5 min. Protein concentration in the eluent was monitored at 280 nm and each protein peak was collected.

A portion of each collected fraction was checked for its trypsin-inhibiting activity in accordance with the procedure of Example 17 (1). Fractions showing the trypsin-inhibiting activity were pooled and lyophilized to obtain a purified sample.

(7) SDS-PAGE

The purified polypeptide sample obtained in the above procedure (6) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(8) Determination of amino acid sequence

A portion of the purified polypeptide sample obtained in the above procedure (7) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6). As the results, it was confirmed that the purified sample is the polypeptide Q19K/Y46D of the present invention (cf. Sequence ID No. 18 of the SEQUENCE LISTING).

Example 11 Production of polypeptide Q19R/Y46D

A polypeptide, Q19R/Y46D, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Arg for the 15 position Gln, as well as Asp for the 42 position Tyr, of the formula 1 sequence counting from its N-terminus was prepared in the following method. (1) Construction of plasmid pM744

PCR was carried out twice using the plasmid pM748 obtained in Example 10 (1) as a template, in accordance with the procedure described in Example 3 (1). In this incidence the first PCR was carried out using the Q19R primer prepared in Example 4 (1) as an antisense primer in stead of the Q19K primer. A finally obtained amplified product of interest having a size of about 380 bp was extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

The thus obtained DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM744. The thus concentrated plasmid pM744 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 340 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer.

The confirmed nucleotide sequence of a region of plasmid pM744 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 37 (cf. Sequence ID No. 19 in the SEQUENCE LISTING).

(2) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM744), was isolated by transforming *E. coli* JE5505 with the plasmid pM744 obtained in the above procedure (1), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3), to recover cultured medium. The resulting cultured medium was concentrated using the aforementioned ultrafiltration membrane, Benchmark GX membrane, thereafter the thus concentrated sample was centrifuged at 10.000 xg for 20 minutes at 4°C to recover the cells.

(3) Isolation and purification of Q19R/Y46D

Inclusion body was obtained in the above procedure (2) in the form of pellet which was subsequently subjected to solubilization and reduction treatments in the same manner as described in Example 10 (4). After the treatment, the sample was refolded in the same manner as described in Example 10 (5) and then purified in the same manner as in Example 10 (6). Finally obtained active fraction was lyophilized to obtain a purified sample.

(4) SDS-PAGE

A portion of the purified polypeptide sample obtained in the above procedure (3) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(5) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (3) was dissolved in 50% ac tic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6). As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (3) is the polypeptide Q19R/Y46D of the present invention (cf. Sequence ID No. 20 of the SEQUENCE LISTING).

Example 12 Production of polypeptide R11Q/Q19K/Y46D

A polypeptide, R11Q/Q19K/Y46D, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Gln for the 11 position Arg, Lys for the 15 position Gln, as well as Asp for the 42 position Tyr, of the formula 1 sequence counting from its N-terminus was prepared in the following method.

(1) Construction of plasmid pM741

PCR was carried out twice using the plasmid pM727 obtained in Example 10 (2) as a template, in accordance with the procedure described in Example 3 (1). In this incidence the first PCR was carried out using a chemically synthesized primer R11Q (Fig. 38) as an antisense primer instead of the Q19K primer. A finally obtained amplified product of interest having a size of about 380 bp was extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

The thus obtained DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM741. The thus constructed plasmid pM741 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 340 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer.

The confirmed nucleotide sequence of a region of plasmid pM741 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 39 (cf. Sequence ID No. 19 in the SEQUENCE LISTING).

(2) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM741), was isolated by transforming *E. coli* JE5505 with the plasmid pM741 obtained in the above procedure (1), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover cultured medium. A portion of the resulting cultured medium was centrifuged to obtain supernatant and to check its trypsin-inhibiting activity in accordance with the procedure of Example 17 (1).

The trypsin-inhibiting activity in the obtained supernatant was 5 times higher than that of *E. coli* JE5505 (pM727). In consideration of the specific activity in bovine trypsin-inhibition, it was confirmed that the substitution of the 7 position counting from N-terminus of the amino acid sequence of formula 1 made an efficient secretion of this polypeptide from a transformant.

The rest of culture mixture was concentrated using the aforementioned ultrafiltration membrane, Benchmark GX membrane. Thereafter the concentrated sample was centrifuged at 10,000 x g for 20 minutes at 4°C to recover the cells.

(3) Isolation and purification of R11Q/Q19K/Y46D

Inclusion body was obtained in the above procedure (2) in the form of pellet which was subsequently subjected to solubilization and reduction treatments in the same manner as described in Example 10 (4). After the treatment, the sample was refolded in the same manner as described in Example 10 (5) and then purified in the same manner as in Example 10 (6). Finally obtained active fraction was lyophilized to obtain a purified sample.

(4) SDS-PAGE

A portion of the purified polypeptide sample obtained in the above procedure (3) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(5) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (3) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6). As the results, it was confirmed that the purified polypeptide sample obtained in the above

procedure (3) is the polypeptide R11Q/Q19K/Y46D of the present invention (cf. Sequence ID No. 22 of the SEQUENCE LISTING).

Example 13 Production of polypeptide R11D/Q19K/Y46D

A polypeptide, R11D/Q19K/Y46D, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Arg for the 11 position Arg, Lys for the 15 position Gln, as well as Asp for the 42 position Tyr, of the formula 1 sequence counting from its N-terminus side was prepared in the following method.

(1) Construction of plasmid pM742

PCR was carried out twice using the plasmid pM727 obtained in Example 10 (2) as a template, in accordance with the procedure described in Example 3 (1). In this incidence the first PCR was carried out using a chemically synthesized primer R11D (Fig. 40) as an antisense primer in stead of the Q19K primer. A finally obtained amplified product of interest having a size of about 380 bp was extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

The thus obtained DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM742. The thus constructed plasmid pM742 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 340 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer.

The confirmed nucleotide sequence of a region of plasmid pM742 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 41 (cf. Sequence ID No. 23 in the SEQUENCE LISTING).

(2) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM742), was isolated by transforming *E. coli* JE5505 with the plasmid pM742 obtained in the above procedure (1), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover cultured medium. A portion of the resulting cultured medium was centrifuged to obtain supernatant and to check its trypsin-inhibiting activity in accordance with the procedure of Example 17 (1).

The trypsin-inhibiting activity in the obtained supernatant was 2 times higher than that of *E. coli* JE5505 (pM727). In consideration of the specific activity in bovine trypsin-inhibition of these polypeptide, it was confirmed that the substitution of the 7 position counting from N-terminus of the amino acid sequence of formula 1 made an efficient secretion of this polypeptide from a transformant.

The rest of cultured medium was concentrated using the aforementioned ultrafiltration membrane, Benchmark GX membrane. Thereafter the concentrated sample was centrifuged at 10,000 x g for 20 minutes at 4°C to recover the cells.

(3) Isolation and purification of R11D/Q19K/Y46D

Inclusion body was obtained in the above procedure (2) in the form of pellet which was subsequently subjected to solubilization and reduction treatments in the same manner as described in Example 10 (4). After the treatment, the sample was refolded in the same manner as described in Example 10 (5) and then purified in the same manner as in Example 10 (6). Finally obtained active fraction was lyophilized to obtain a purified sample.

(4) SDS-PAGE

A portion of the purified polypeptide sample obtained in the above procedure (3) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(5) Determination of amino acid sequence

The purifi d polypeptide sample obtained in the above procedure (3) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6). As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (3) is the polypeptide R11D/Q19K/Y46D of the present invention (cf. Sequence ID No. 24 of the SEQUENCE LISTING).

Example 14 Production of polypeptide R11L/Q19K/Y46D

A polypeptide, R11L/Q19K/Y46D, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Leu for the 11 position Arg, Lys for the 15 position Gln, as well as Asp for the 42 position Tyr, of the formula 1 sequence counting from its N-terminus side was prepared in the following method.

(1) Construction of plasmid pM743

PCR was carried out twice using the plasmid pM727 obtained in Example 10 (2) as a template, in accordance with the procedure described in Example 3 (1). In this incidence the first PCR was carried out using a chemically synthesized primer R11L (Fig. 42) as an antisense primer instead of the Q19K primer. A finally obtained amplified product of interest having a size of about 380 bp was extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

The thus obtained DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM743. The thus constructed plasmid pM743 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 340 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer.

The confirmed nucleotide sequence of a region of plasmid pM743 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 43 (cf. Sequence aid No. 25 in the SEQUENCE LISTING).

(2) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM743), was isolated by transforming *E. coli* JE5505 with the plasmid pM743 obtained in the above procedure (1), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover cultured medium.

The resulted cultured medium was concentrated using the aforementioned ultrafiltration membrane, Benchmark GX membrane. Thereafter the concentrated sample was centrifuged at 10,000 x g for 20 minutes at 4°C to recover the cells.

(3) Isolation and purification of R11L/Q19K/Y46D

Inclusion body was obtained in the above procedure (2) in the form of pellet which was subsequently subjected to solubilization and reduction treatments in the same manner as described in Example 10 (4). After the treatment, the sample was refolded in the same manner as described in Example 10 (5), and then purified in the same manner as in Example 10 (6). Finally obtained active fraction was lyophilized to obtain a purified sample.

(4) SDS-PAGE

A portion of the purified polypeptide sample obtained in the above procedure (3) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(5) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (3) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6). As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (3) is the polypeptide R11L/Q19K/Y46D of the present invention (cf. Sequence ID No. 26 of the SEQUENCE LISTING).

Example 15 Production of polypeptide R11E/Q19K/Y46E

A polypeptide, R11E/Q19K/Y46E, having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Glu for the position 11 Arg, Lys for the 15 position Gln, as well as Glu for the 42 position Tyr, of the formula 1 sequence counting from its N-terminus side was prepared in the following method.

(1) Construction of plasmid pM721

PCR was carried out twice in accordance with the procedure described in Example 3 (1) using a plasmid pM727 as a template. In this instance, the first PCR was carried out using Y46E prepared in Example 2 (1) and pBRBamHI primer prepared in Example 3 (1) as sense and antisense primers, respectively. An amplified DNA fragment obtained by the first PCR having a size of about 120 bp was then extracted and purified by means of phenol treatment and ethanol precipitation and then dissolved in TE buffer. Thereafter, second PCR was carried out using the thus dissolved DNA fragment as an antisence primer and the *Hind*III prepared in Example 3 (1) primer as a sense primer. Thereby a finally obtained amplified product of interest having a size of about 380 bp was extracted and purified by means of phenol treatment and ethanol precipitation. Then, the DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM721.

(2) Construction of plasmid pM738

PCR was carried out twice using the thus plasmid pM721, in accordance with the procedure described in Example 3 (1). In this incidence, the first PCR was carried out using R11E primer prepared in Example 6 (1) as an antisence primer instead of the Q19K primer. A finally obtained amplified product of interest having a size of about 380 bp was extracted and purified by means of phenol treatment and ethanol precipitation to obtain the DNA fragment of the present invention.

Thus obtained DNA fragment was inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM738. The thus constructed plasmid pM738 was double-digested with *Hind*III and *Bam*HI, and a DNA fragment of interest having a size of about 340 bp was extracted and purified. Thereafter, sequencing was carried out in the same manner as described in Example 2 (2) using the aforementioned DNA sequencer.

The confirmed nucleotide sequence of a region of plasmid pM738 from its *Hind*III site to *Bam*HI site containing the novel DNA fragment of the present invention and its corresponding amino acid sequence are shown in Fig. 44 (Sequence ID No. 27 of the SEQUENCE LISTING).

(3) Preparation and culturing of transformant

A transformant, *E. coli* JE5505 (pM738), was isolated by transforming *E. coli* JE5505 with the plasmid pM738 obtained in the above procedure (2), in accordance with the Hanahan's method. The thus isolated transformant was subsequently cultured in the same manner as in Example 3 (3) to recover cultured medium. A portion of the resulting cultured medium was centrifuged to obtain supernatant and to check its trypsin-inhibiting activity in accordance with the procedure of Example 17 (1).

The trypsin-inhibiting activity in the obtained supernatant was about 5 times higher than that of *E. coli* JE5505 (pM721) which was transformed by the plasmid pM 721 obtained in above (1). In consideration of the specific activity of these polypeptides in bovine trypsin-inhibition, it was confirmed that the substitution of the 7 position counting from N-terminus of the amino acid sequenc formula 1 made an ffici nt secretion of this polypeptide from a transformant.

The rest of cultured medium was concentrated using the aforemention d ultrafiltration membrane, Benchmark GX membrane. Thereafter the concentrated sample was centrifuged at 10,000 x g for 20 minutes at 4°C to recover the c lls.

(4) Isolation and purification of R11E/Q19K/Y46E

Inclusion body was obtained in the above procedure (3) in the form of pellet which was subsequently subjected to solubilization and reduction treatments in the same manner as described in Example 10 (4). After the treatment, the sample was refolded in the same manner as described in Example 10 (5) and then purified in the same manner as in Example 10 (6). Finally obtained active fraction was lyophilized to obtain a purified sample.

(5) SDS-PAGE

A portion of the purified polypeptide sample obtained in the above procedure (3) was subjected to SDS-PAGE in accordance with the procedure described in Example 2 (5). After the electrophoresis, staining was carried out with the aforementioned commercial silver staining kit. The purified sample showed a single band by SDS-PAGE.

(6) Determination of amino acid sequence

The purified polypeptide sample obtained in the above procedure (4) was dissolved in 50% acetic acid solution and its amino acid sequence was determined in accordance with the procedure described in Example 2 (6). As the results, it was confirmed that the purified polypeptide sample obtained in the above procedure (3) is the aimed polypeptide R11E/Q19K/Y46E of the present invention (cf. Sequence ID No. 28 of the SEQUENCE LISTING).

Example 16 Production of polypeptide R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D

Polypeptides R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D having an amino acid sequence derived from the aforementioned formula 1 amino acid sequence by substituting Asn, Ser and Ala for the 7 position Arg, respectively, in addition to substituting Lys for the 15 position Gln and Asp for the 42 position Tyr of the formula 1 sequence counting from its N-terminus side were prepared by the following method.

That is, DNA fragments which have a nucleotide sequence coding the amino acid sequence of polypeptides R11N/Q19K/Y46D, R11S/Q19K/Y46D, R11A/Q19K/Y46D (cf. Sequence ID No.29, 30 and 31) respectively, were prepared by the site-directed mutagenesis, the method of Landt et. al. The thus obtained DNA fragments were inserted into the aforementioned plasmid pM463 in accordance with the procedure described in Example 3 (2) to obtain the expression plasmid pM764, pM765, pM767 and sequencing was carried out in the same manner as described in Example 2 (2).

The confirmed nucleotide sequences of each region of plasmid pM764, pM765 and pM767 from its HindIII site to BamHI site containing the novel DNA fragments of the present invention and those corresponding amino acid sequences are shown in Sequence ID No.32, 33 and 34 in the SEQUENCE LISTING. *E. coli* JE5505 were transformed with the plasmid pM764, pM765 and pM767 respectively, in accordance with the Hanahan's method. And then transformants, *E. coli* JE5505 (pM764), *E. coli* JE5505 (pM765), *E. coli* JE5505 (pM767) were isolated and cultured in the same manner as described in Example 3 (3). A portion of the cultured medium of each transformant was centrifuged to obtain supernatant and to check its trypsin-inhibiting activity in accordance with the procedure of Example 17 (1).

All of the obtained supernatant showed about 4 times higher trypsin-inhibiting activity in comparison with that of *E. coli* JE5505 (pM727). In consideration of the specific activity of these polypeptides in bovine trypsin-inhibition, it was confirmed that these substitutions of 7 position counting from N-terminus of the amino acid sequence formula 1 made an efficient secretion of this polypeptide from a transformant.

Example 17 Measurement of enzyme-inhibiting activities

Trypsin-, FXa- and elastase-inhibiting activities of the novel polypeptide of the present invention were measured in the following manner, using polypeptide samples Y46E, Q19K, Q19R, Q19K/Y46E, R11E/Y46E, Y46E-AN, Q19K-AN, Q19R/Y46E, Q19K/Y46D, Q19R/Y46D, R11Q/Q19K/Y46D,

R11D/Q19K/Y46D, R11L/Q19K/Y46D, R11E/Q19K/Y46E, R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D obtained in Example 2 (4), Example 3 (4), Example 4 (4), Example 5 (4), Example 6 (4), Example 7 (3), Example 8 (3), Example 9 (4), Example 10 (6), Example 11 (3), Example 12 (3), Example 13 (3), Example 14 (3), Example 15 (4) and Example 16, respectively.

(1) Trypsin - inhibiting activity

The purified polypeptide of the present invention was dissolved in 100 µl of distilled water, and the solution was serially diluted with a 0.1% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8) for use in the activity measurement. Measurement of the trypsin - inhibiting activity in the thus prepared test sample was carried out in accordance with the Kassell's method (Kassell,B. *et al.*, *Methods in Enzymology*, vol.19, pp.844 - 852, 1970) using a synthetic substrate S - 2444 (Daiichi Pure Chemicals Co., Ltd.).

Bovine trypsin (Type XIII, Sigma Chemical Co.) was dissolved in 0.001 M HCl to a final concentration of 13.600 BAEEU/ml and the solution was further diluted with the 0.1% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8) to prepare 1.2 BAEEU/ml of trypsin solution. Separately from this, 2 mM solution of the synthetic substrate S - 2444 was prepared by dissolving it in distilled water. Next, 100 µl of the test sample was mixed with 100 µl of the trypsin solution. After incubating statically at 37 ° C for 10 minutes, 50 µl of the S - 2444 solution was added to start the reaction. The reaction was carried out at 37 ° C for 15 minutes and then stopped by adding 50 µl of 50% acetic acid solution to the reaction mixture. Thereafter, absorbance at a wave length of 405 nm was measured using a spectrophotometer. In this instance, in order to eliminate absorbancy of various contents in the reaction mixture other than the reaction product, a blank solution was prepared by mixing 100 µl of the bovine trypsin solution with 50 µl of 50% acetic acid and then with 100 µl of each test sample and 50 µl of the S - 2444 solution. As the results, it was confirmed that each of these polypeptides Q19K, Q19R, Q19K/Y46E, R11E/Y46E, Y46E - AN, Q19K - AN, Q19R/Y46E, Q19K/Y46D, Q19R/Y46D, R11Q/Q19K/Y46D, R11D/Q19K/Y46D, R11L/Q19K/Y46D, R11E/Q19K/Y46E, R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D can inhibit bovine trypsin in a concentration - dependent manner.

(2) Human trypsin - inhibiting activity

The purified polypeptide of the present invention was dissolved in 100 µl of distilled water. Concentration of polypeptide in the thus prepared solution was determined based on its bovine trypsin - inhibiting activity measured in accordance with the above procedure (1) using UTI (Mochida Pharmaceutical Co., Ltd.; cf. H. Ohnishi *et al.*, *Nippon Yakurigaku Zasshi*, vol.85, pp.1 - 6, 1985) as a standard. The polypeptide solution was then diluted to various concentration levels with 0.03% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8) for use in the activity measurement. Separately from this, human trypsin (Calbiochem) was dissolved in 1 mM HCl to a concentration of 1,000 SU/ml and the solution was further diluted with the 0.03% BSA/0.2 M triethanolamine - HCl buffer (pH 7.8) to prepare a 1.25 SU/ml solution of human trypsin. Thereafter, human trypsin - inhibiting activity was measured in the same manner as described in the above procedure (1) using the aforementioned synthetic substrate S - 2444. As the results, it was confirmed each of these polypeptides Q19K, Q19R, Q19K/Y46E, R11E/Y46E, Y46E - AN, Q19K - AN, Q19R/Y46E, Q19K/Y46D, Q19R/Y46D R11Q/Q19K/Y46D, R11D/Q19K/Y46D, R11L/Q19K/Y46D, R11E/Q19K/Y46E, R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D can inhibit human trypsin in a concentration - dependent manner.

(3) FXa - inhibiting activity

The purified polypeptide of the present invention was dissolved in 100 µl of distilled water. Polypeptide concentration of this solution was determined based on its bovine trypsin - inhibiting activity measured in accordance with the above procedure (1) using the aforementioned UTI as a standard. The thus prepared solution was then diluted to various concentration levels with 0.1% BSA/150 mM NaCl/5 mM $CaCl_2$/50 mM Tris - HCl buffer (pH 8.3) for use in the activity measurement. Separately from this, a polypeptide TN70 (cf. Japanese Patent Application No. 3 - 325220) and polypeptide AN68 were purified respectively in accordance with the procedure of Example 2 (4) from a culture supernatant of *E. coli* JE5505(pM552) (deposition No., FERM BP - 3561) and *E. coli* JE5505(pM594) obtained by transforming *E. coli* JE5505 with the aforementioned plasmid pM594 obtain in Example 4 (1). The thus purified polypeptide TN70 and AN68 were diluted to various concentration levels to be used as a control solution.

The amino acid sequence of polypeptide TN 70 was repres nted in Formula 3, and amino acid sequence of polypeptide AN68 was represented below, in Formula 5.

Using a synthetic compound S-2222 (Daiichi Pur Chemicals Co., Ltd.) as a substrate, FXa-inhibiting activities in the thus prepared test sample solution and control solution were measured in accordance with the method of Ohno *et al.* (Ohno H. *et al.*, *Thromb. Res.*, vol.19, pp.579 - 588, 1980) as follows. Firstly, human FXa (American Diagnostica Inc.) was dissolved in distilled water to a final concentration of 10 PEU/ml and the solution was further diluted with the aforementioned 0.1% BSA/150 mM NaCl/5 mM $CaCl_2$/50 mM Tris-HCl buffer (pH 8.3) to prepare 0.1 PEU/ml of FXa solution. Separately from this, 4 mM solution of S-2222 was prepared by dissolving it in distilled water and the solution was further diluted with the 0.1% BSA/150 mM NaCl/5 mM $CaCl_2$/50 mM Tris-HCl buffer (pH 8.3) to obtain 2 mM solution of S-2222.

Next, 25 µl of the test sample or control solution was mixed with 100 µl of the 0.1% BSA/150 mM NaCl/5 mM $CaCl_2$/50 mM Tris-HCl buffer (pH 8.3) and 25 µl of the FXa solution. After incubating statically at 37°C for 10 minutes, 100 µl of the S-2222 solution was added to start the reaction. The reaction was carried out at 37°C for 30 minutes and then stopped by adding 50 µl of 50% acetic acid solution to the reaction mixture. Thereafter, absorbance at a wave length of 405 nm was measured using a spectrophotometer. In this instance, in order to eliminate absorbancy of various contents in the reaction mixture other than the reaction product, a blank solution was prepared by mixing 25 µl of the FXa solution with 50 µl of 50% acetic acid and then with 25 µl of each test sample or the control solution, 100 µl of the 0.1% BSA/150 mM NaCl/5 mM $CaCl_2$/50 mM Tris-HCl buffer (pH 8.3) and with 100 µl of the S-2222 solution.

Formula 5

Ala Ala Cys Asn Leu Pro Ile Val Arg Gly
Pro Cys Arg Ala Phe Ile Gln Leu Trp Ala
Phe Asp Ala Val Lys Gly Lys Cys Val Leu
Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly
Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg
Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
Glu Glu Leu Leu Arg Phe Ser Asn

The results are shown in Fig. 45 to Fig. 53. In these figures, polypeptide concentration in the reaction solution was expressed as its bovine trypsin-inhibiting activity. Also, residual human FXa activity was expressed by percentage based on the absorbance of a control reaction mixture in which 25 µl of the 0.1% BSA/150 mM NaCl/5 mM $CaCl_2$/50 mM Tris-HCl buffer (pH 8.3) was used instead of the test sample.

Fig. 45 shows the novel polypeptide Y46E of the present invention has significantly high FXa-inhibiting activity in comparison with the polypeptide TN70 used as a control. In other words, it has an FXa-inhibiting activity which is about seven times higher than that of the polypeptide TN70.

Fig. 46 shows the novel polypeptide Q19K of the present invention has significantly high FXa-inhibiting activity in comparison with the polypeptide TN70 used as a control. In other words, it has an FXa-inhibiting activity which is about five times higher than that of the polypeptide TN70.

Fig. 47 shows the novel polypeptide Q19R of the present invention has significantly high FXa-inhibiting activity in comparison with the polypeptide AN68 used as a control. In other words, it has an FXa-inhibiting activity which is about six times higher than that of the polypeptide AN68.

Fig. 48 shows the novel polypeptide Q19K/Y46E of the present invention has significantly high FXa-inhibiting activity in comparison with the polypeptide AN68 used as a control. In other words, it has an FXa-inhibiting activity which is about ten times higher than that of the polypeptide AN68.

Fig. 49 shows the novel polypeptide R11E/Y46E of the present invention has significantly high FXa-inhibiting activity in comparison with the polypeptide AN68 used as a control. In other words, it has an FXa-inhibiting activity which is about three times higher than that of the polypeptide AN68.

Fig. 50 shows the novel polypeptide Q19R/Y46E of the present invention has an FXa-inhibiting activity which is about ten times higher than that of the polypeptide AN68.

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Fig. 51 shows th novel polypeptides Q19K/Y46D and Q19R/Y46D of the present invention have significantly high FXa-inhibiting activity in comparison with the polypeptide AN68 used as a control. In other words, polypeptide Q19K/Y46D has an FXa-inhibiting activity which is about twenty times higher than that of the polypeptide AN68. And polypeptide Q19R/Y46D has an FXa-inhibiting activity which is about 18 times higher than that of the polypeptide AN68.

Fig. 52 shows the novel polypeptides R11Q/Q19K/Y46D, R11L/Q19K/Y46D, R11D/Q19K/Y46D of the present invention have significantly high FXa-inhibiting activity in comparison with the polypeptide AN68 used as a control. In other words, polypeptide R11Q/Q19K/Y46D of the present invention has an FXa-inhibiting activity which is about twenty times higher than that of the polypeptide AN68. The polypeptide R11D/Q19K/Y46D has an FXa-inhibiting activity which is about 5 times higher than that of the polypeptide AN68. The polypeptide R11L/Q19K/Y46D has an FXa-inhibiting activity which is about 16 times higher than that of the polypeptide AN68.

Fig. 53 shows the novel polypeptide R11E/Q19K/Y46E of the present invention has significantly high FXa-inhibiting activity in comparison with the polypeptide AN68 used as a control. In other words, it has an FXa-inhibiting activity which is about 13 times higher than that of the polypeptide AN68.

(4) Human elastase-inhibiting activity

The purified polypeptide of the present invention was dissolved in 100 μl of distilled water. Concentration of this solution was determined based on its bovine trypsin-inhibiting activity using the UTI as a standard. The solution was then diluted to various concentration levels with 0.1% BSA/27 mM $CaCl_2$/133 mM Tris-HCl buffer (pH 7.5) for use in the activity measurement. The aforementioned AN68 was also diluted in the same manner for use as a control. Human elastase-inhibiting activities in the test samples and the positive control were measured in accordance with the method of Ogawa *et al.* (Ogawa,M. *et al.*, *Res. Commun. Chem. Pathol. Pharmacol.*, vol.55, pp.271 - 274, 1987) using a synthetic compound STANA (Peptide Institute Inc.) as a substrate.

Human nuetrophil elastase (CALBIOCHEM) was dissolved in 27 mM $CaCl_2$/133 mM Tris-HCl buffer (pH 7.5) to a concentration of 6U/ml and the solution was further diluted with 0.1% BSA/27 mM $CaCl_2$/133 mM Tris-HCl buffer (pH 7.5) to prepare 0.24U/ml of elastase solution. 100 mM STANA solution was prepared by dissolving it in N-methyl-2-pyrrolidone and the solution was further diluted with 0.1% BSA/27 mM $CaCl_2$/133 mM Tris-HCl buffer (pH 7.5) to prepare 20 mM STANA solution..

Next, 50 μl of the test sample or positive control was mixed with 50 μl of the elastase solution and 50 μl of 0.1% BSA/27 mM $CaCl_2$/133 mM Tris-HCl buffer (pH 7.5). After incubation at 37°C for 10 minutes, 50 μl of the STANA solution was added to the mixture to start the reaction. The reaction was carried out at 37°C for 20 minutes and then stopped by adding 50 μl of 50% acetic acid solution to the reaction mixture. Thereafter, absorbance at a wave length of 405 nm was measured using a spectrophotometer.

In this instance, in order to eliminate absorbance of various solutions in the reaction product, a blank solution was prepared by mixing 50 μl of the elastase solution with 50 μl of 50% acetic acid and then with 50 μl of each test sample or the positive control, 50 μl of 0.1% BSA/27 mM $CaCl_2$/133 mM Tris-HCl buffer (pH 7.5) and with 50 μl of the STANA solution.

The results are shown in Fig. 54 and 55. In these figure, polypeptide concentration in the reaction solution was expressed as its bovine trypsin-inhibiting activity. Also, remaining human elastase activity was expressed by percentage based on the absorbance of a control reaction mixture in which 50 μl of the 0.1% BSA/27mM $CaCl_2$/133mM Tris-HCl buffer (pH 7.5) was used instead of the test sample or the control.

Fig. 54 shows the novel polypeptide R11Q/Q19K/Y46D, R11D/Q19K/Y46D and R11L/Q19K/Y46D of the present invention have significantly high elastase-inhibiting activity in comparison with the polypeptide AN68 used as a control and the polypeptide Q19K/Y46D which was obtained Example 10 (6). In other words, the polypeptide R11Q/Q19K/Y46D has an Elastase-inhibiting activity which is about 3 times higher than that of the polypeptide AN68 and the polypeptide Q19K/Y46D. The polypeptide R11D/Q19K/Y46D has an Elastase-inhibiting activity which is about 3 times higher than that of the polypeptide AN68 and the polypeptide Q19K/Y46D. And the polypeptide R11L/Q19K/Y46D has an Elastase-inhibiting activity which is about 2 times higher than that of the polypeptide AN68 and the polypeptide Q19K/Y46D.

Fig. 55 shows the novel polypeptide R11E/Q19K/Y46E of the present invention has significantly high Elastase-inhibiting activity in comparison with the polypeptide AN68 used as a control and the polypeptide Q19K/Y46E which was purified from the cultured medium of *E. coli* JE5505(pM721) transformed by plasmid pM721 obtain d Example 15 (1). In other words, it has an Elastase-inhibiting activity which is about 5 times higher than that of the polypeptide AN68 and 6 times higher than that of the polypeptide Q19K/Y46E.

Example 18 Safety

Safety of the novel polypeptide of the present invention was confirmed by the following procedures (1) and (2).

(1) Polypeptide Y46E, Q19K, Q19R, Q19K/Y46E, R11E/Y46E, Y46E-AN, Q19K-AN and Q19R/Y46E were prepared from the cultured medium of transformants in accordance with the procedure in Example 2 (4).

Each of the thus prepared ten purified polypeptide samples was dissolved in physiological saline and the resulting solution was applied to PIROZALTO UNIT (molecular weight cutoff of 20,000, Saltorius) in order to remove lipopolysaccharide (to be referred to as "LPS" hereinafter).

Wister Rats were prepared for one week in advance and then divided into administration groups and control groups, each test group including 10 males or females. Each of the polypeptide solutions prepared above was administered to rats of each administration group by intravenous injection with a dose of 100 mg/kg/day, and appearance of symptoms and changes in their body weights were observed for one week. In this instance, physiological saline was administered to the control group.

As the results, significant side effect was not found in any of the polypeptide-administered groups, and survival rates and body weight changes in the administration groups were the same as those in the control groups.

(2) Polypeptide Q19K/Y46D, Q19R/Y46D, R11Q/Q19K/Y46D, R11D/Q19K/Y46D, R11L/Q19K/Y46D, R11E/Q19K/Y46E, R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D were prepared by purification from the cultured medium of transformants in accordance with the procedure in Example 10 (6).

Each of purified polypeptide was dissolved in physiological saline and applied to OMEGACEL (molecular weight cutoff of 100,000, Filtoron) in order to remove LPS.

Wister Rats were prepared for one week in advance and then divided into administration groups and control groups, each group including 10 males or females. Each of the polypeptide solutions prepared above was administered to rats of each administration group by intravenous injection with a dose of 10 mg/kg/day. While, physiological saline was administered to rats of each control group. An appearance of symptoms and changes in their body weights were observed for a week. As the results, significant side effect was not found in any of the polypeptide-administered groups, and survival rates and body weight changes in the administration groups were the same as those in the control groups.

Example 19 pharmaceutical preparations

Polypeptides Y46E, Q19K, Q19R, Q19K/Y46E, R11E/Y46E, Y46E-AN, Q19K-AN and Q19R/Y46E were prepared from the cultured medium of transformants in accordance with the procedure in Example 2 (4).

Each of the thus prepared ten purified polypeptide samples was dissolved in physiological saline and the resulting solution was applied to a PIROZALTO unit in order to remove LPS.

Polypeptides Q19K/Y46D, Q19R/Y46D, R11Q/Q19K/Y46D, R11D/Q19K/Y46D, R11E/Q19K/Y46E, R11N/Q19K/Y46D, R11S/Q19K/Y46D and R11A/Q19K/Y46D were prepared by purification from the cultured medium of transformants in accordance with the procedure in Example 2 (4) or Example 10 (6).

Each of purified polypeptides were dissolved in distilled water and then applied to an aforementioned membrane, OMEGACEL in order to remove LPS. Thereafter, each of the thus prepared polypeptide solutions was dried under a reduced pressure and then made into pharmaceutical preparations in accordance with the following procedures (1) and (2).

(1) Each of the thus prepared polypeptide samples was dissolved, to a final concentration of 2 mg/ml, in 1/15 M phosphate buffer (pH 7.4) containing 0.1% (w/v) of pyrogen-free gelatin which has been prepared using distilled water for injection use. To the resulting solution of each polypeptide sample were added sodium chloride to a final concentration of 75 mM and mannitol to a final concentration of 2% (w/v). The thus prepared sample solution was sterilized by filtration through an aseptic membrane filter having a pore size of 0.22 μm (Disposable Sterile Filter System, Corning) and dispensed in 5 ml aliquots into glass vessels.

(2) Each of the thus prepared polypeptide samples was dissolved, to a final concentration of 2 mg/ml, in 0.01 M phosphate buffer (pH 7.4) containing 0.14 M sodium chloride which has been prepared using distilled water for injection use. To the resulting solution of each polypeptide sample was added human serum albumin to a final concentration of 1% (w/v). The thus prepared sample solution was sterilized by filtration through the aforementioned aseptic membrane filter having a pore size of 0.22 μm (Disposable

Sterile Filter System) and dispensed in 5 ml aliquots into glass vessels. Thereafter, the thus disp nsed samples were freez - dried and sealed.

Thus, it is apparent that there have been provided, in accordance with the present invention, novel polypeptides and processes for the production thereof. The present invention also provides DNA fragments which encode the novel polypeptides, vectors containing the DNA fragments and transformants transformed with the DNA fragments or the vectors. This invention also provides drug compositions which contain the novel polypeptides as the active ingredient, as well as enzyme inhibition processes in which the novel polypeptides are used.

The polypeptide of this invention can be used not only as an active ingredient of a drug composition but also for the purpose of preventing blood coagulation on the surface of medical instruments such as artificial blood vessels, artificial organs, catheters and the like, by binding or adsorbing the polypeptide to the surface of instruments making use of a cross - linking agent and the like. In consequence, this invention opens a new way for preventing and treating various diseases including protease - related diseases such as infestation, multiple - organ failure, shock, pancreatitis, disseminated intravascular coagulation syndrome, ischemic heart disease, nephritis, hepatic cirrhosis, re - obstruction at the time of blood circulation recon - structive operation, edema caused by increased vascular permeability, adult respiratory distress syndrome, rheumatoid arthritis, arthritis and allergic diseases.

Although some preferred embodiments have been described, many modifications and variations may be made thereto in the light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

[SEQUENCE LISTING]

SEQ ID NO: 1

SEQUENCE LENGTH: 313

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM575B)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC ACC GTC   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Thr Val
             -10                 - 5                   1

 GCC GCC TGC AAT CTC CCC ATA GTC CGG GGC CCC TGC CGA GCC TTC  140
 Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
           5                  10                  15

 ATC CAG CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC  185
 Ile Gln Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
          20                  25                  30

 TTC CCC TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA  230
 Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser
          35                  40                  45

 GAG AAG GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT  275
 Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
          50                  55                  60

 GAG GAG CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  313
 Glu Glu Leu Leu Arg Phe Ser Asn
          65                  70
```

SEQ ID NO: 2

SEQUENCE LENGTH: 70

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Thr Val Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg
 1               5                  10                  15

Ala Phe Ile Gln Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys
            20                  25                  30

Val Leu Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe
        35                  40                  45

Glu Ser Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp
    50                  55                  60

Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn
65                  70
```

SEQ ID NO: 3

SEQUENCE LENGTH: 313

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*
STRAIN: JE5505 (pM576)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GAC GAC    95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Asp Asp
            -10                 - 5                   1

GCC GCC TGC AAT CTC CCC ATA GTC CGG GGC CCC TGC CGA GCC TTC   140
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
          5                   10                  15

ATC AAG CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC   185
Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
         20                  25                  30

TTC CCC TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC TAC TCA   230
Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser
         35                  40                  45

GAG AAG GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT   275
Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
         50                  55                  60

GAG GAG CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC   313
Glu Glu Leu Leu Arg Phe Ser Asn
         65                  70
```

SEQ ID NO: 4

SEQUENCE LENGTH: 70

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Asp Asp Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg
  1               5                  10                  15

Ala Phe Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys
                20                  25                  30

Val Leu Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe
                35                  40                  45

Tyr Ser Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp
                50                  55                  60

Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn
                65                  70
```

SEQ ID NO: 5

SEQUENCE LENGTH: 313

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM735)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCG GCC   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Ala
             -10                 - 5                   1

 TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC CGT  140
 Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Arg
           5                  10                  15

 CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC  185
 Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
          20                  25                  30

 TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC TAC TCA GAG AAG  230
 Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys
          35                  40                  45

 GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG  275
 Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
          50                  55                  60

 CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  307
 Leu Leu Arg Phe Ser Asn
          65          68
```

SEQ ID NO: 6

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Arg Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser
        35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
    50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
65                  68
```

SEQ ID NO: 7

SEQUENCE LENGTH: 307

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM736)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                 -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCG GCC    95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Ala
            -10                 - 5                   1

TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC AAG   140
Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Lys
          5                  10                  15

CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC   185
Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
         20                  25                  30

TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG   230
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys
         35                  40                  45

GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG   275
Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
         50                  55                  60

CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  307
Leu Leu Arg Phe Ser Asn
         65          68
```

SEQ ID NO: 8

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser
        35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
    50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
65                  68
```

SEQ ID NO: 9

SEQUENCE LENGTH: 307

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM726B)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCG GCC    95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Ala
             -10                 - 5                   1

 TGT AAT CTA CCA ATA GTC GAA GGC CCC TGC CGA GCC TTC ATC CAG   140
 Cys Asn Leu Pro Ile Val Glu Gly Pro Cys Arg Ala Phe Ile Gln
           5                   10                  15

 CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC   185
 Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
          20                  25                  30

 TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG   230
 Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys
          35                  40                  45

 GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG   275
 Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
          50                  55                  60

 CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  307
 Leu Leu Arg Phe Ser Asn
          65          68
```

SEQ ID NO: 10

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Glu Gly Pro Cys Arg Ala Phe
  1               5                  10                  15
Ile Gln Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
             20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser
             35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
             50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
             65              68
```

SEQ ID NO: 11

SEQUENCE LENGTH: 307

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM575C)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCG GCC   95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Ala
            -10                 - 5                   1

TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC CAG  140
Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln
          5                  10                  15

CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC  185
Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
         20                  25                  30

TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG  230
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys
         35                  40                  45

GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG  275
Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
         50                  55                  60

CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  307
Leu Leu Arg Phe Ser Asn
         65          68
```

SEQ ID NO: 12

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Gln Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser
        35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
    50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
65                  68
```

SEQ ID NO: 13

SEQUENCE LENGTH: 307

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM576B)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCG GCC   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Ala
             -10                 - 5                   1

 TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC AAG  140
 Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Lys
           5                  10                  15

 CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC  185
 Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
         20                  25                  30

 TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC TAC TCA GAG AAG  230
 Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys
         35                  40                  45

 GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG  275
 Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
         50                  55                  60

 CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  307
 Leu Leu Arg Phe Ser Asn
         65          68
```

SEQ ID NO: 14

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 15

SEQUENCE LENGTH: 307

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM737B)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCG GCC    95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Ala
            -10                 - 5                   1

TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC CGT   140
Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Arg
          5                  10                  15

CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC   185
Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
         20                  25                  30

TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG   230
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys
         35                  40                  45

GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG   275
Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
         50                  55                  60

CTG CTG CGC TTC TCC AAC TGACAACTGG ATCC  307
Leu Leu Arg Phe Ser Asn
         65          68
```

SEQ ID NO: 16

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Arg Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 17

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM727)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG   95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
            -10                                 -1   1

CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA  140
Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
          5                   10                  15

CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA  185
Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
         20                  25                  30

TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC  230
Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
         35                  40                  45

TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA  275
Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
         50                  55                  60

GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC  320
Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
         65                  70                  75

TTC TCC AAC TGACAACTGG ATCC  343
Phe Ser Asn
         80
```

SEQ ID NO: 18
SEQUENCE LENGTH: 68
SEQUENCE TYPE: amino acid
MOLECULE TYPE: peptide
SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
        35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
    50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
65                  68
```

SEQ ID NO: 19

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*
STRAIN: JE5505 (pM744)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                 -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG    95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
             -10                                 -1   1

 CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA   140
 Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
           5                  10                  15

 CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC CGT CTC TGG GCA   185
 Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Arg Leu Trp Ala
          20                  25                  30

 TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC   230
 Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
          35                  40                  45

 TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA   275
 Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
          50                  55                  60

 GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC   320
 Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
          65                  70                  75

 TTC TCC AAC TGACAACTGG ATCC  343
 Phe Ser Asn
          80
```

SEQ ID NO: 20

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Arg Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
        35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
    50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
65                  68
```

SEQ ID NO: 21

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM741)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
             -10                                 -1   1

 CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA  140
 Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
           5                  10                  15

 CCA ATA GTC CAG GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA  185
 Pro Ile Val Gln Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
          20                  25                  30

 TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC  230
 Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
          35                  40                  45

 TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA  275
 Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
          50                  55                  60

 GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC  320
 Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
          65                  70                  75

 TTC TCC AAC TGACAACTGG ATCC  343
 Phe Ser Asn
          80
```

SEQ ID NO: 22

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Gln Gly Pro Cys Arg Ala Phe
  1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
             20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
             35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
             50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
             65              68
```

SEQ ID NO: 23

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*
STRAIN: JE5505 (pM742)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
             -10                                 -1   1

 CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA  140
 Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
           5                  10                  15

 CCA ATA GTC GAT GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA  185
 Pro Ile Val Asp Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
          20                  25                  30

 TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC  230
 Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
          35                  40                  45

 TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA  275
 Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
          50                  55                  60

 GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC  320
 Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
          65                  70                  75

 TTC TCC AAC TGACAACTGG ATCC  343
 Phe Ser Asn
          80
```

SEQ ID NO: 24

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Asp Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 25

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*
STRAIN: JE5505 (pM743)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
            -10                                 -1   1

 CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA  140
 Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
           5                   10                  15

 CCA ATA GTC CTG GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA  185
 Pro Ile Val Leu Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
          20                  25                  30

 TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC  230
 Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
          35                  40                  45

 TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA  275
 Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
          50                  55                  60

 GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC  320
 Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
          65                  70                  75

 TTC TCC AAC TGACAACTGG ATCC  343
 Phe Ser Asn
          80
```

SEQ ID NO: 26

SEQUENCE LENGTH: 68

SEQUENCE TYPE: amino acid

MOLECULE TYPE: peptide

SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Leu Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
        35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
    50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
65                  68
```

SEQ ID NO: 27

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM738)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG    95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
            -10                                 -1   1

CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA   140
Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
          5                   10                  15

CCA ATA GTC GAA GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA   185
Pro Ile Val Glu Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
         20                  25                  30

TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC   230
Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
         35                  40                  45

TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG GAG TGC AGA   275
Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys Glu Cys Arg
         50                  55                  60

GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC   320
Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
         65                  70                  75

TTC TCC AAC TGACAACTGG ATCC  343
Phe Ser Asn
         80
```

SEQ ID NO: 28
SEQUENCE LENGTH: 68
SEQUENCE TYPE: amino acid
MOLECULE TYPE: peptide
SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Glu Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 29
SEQUENCE LENGTH: 68
SEQUENCE TYPE: amino acid
MOLECULE TYPE: peptide
SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Asn Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 30
SEQUENCE LENGTH: 68
SEQUENCE TYPE: amino acid
MOLECULE TYPE: peptide
SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Ser Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 31
SEQUENCE LENGTH: 68
SEQUENCE TYPE: amino acid
MOLECULE TYPE: peptide
SEQUENCE

```
Ala Ala Cys Asn Leu Pro Ile Val Ala Gly Pro Cys Arg Ala Phe
 1               5                  10                  15

Ile Lys Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu
            20                  25                  30

Phe Pro Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser
            35                  40                  45

Glu Lys Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp
            50                  55                  60

Glu Glu Leu Leu Arg Phe Ser Asn
            65          68
```

SEQ ID NO: 32

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM764)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Th  Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG   95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
            -10                                 -1   1

CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA  140
Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
          5                   10                  15

CCA ATA GTC AAC GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA  185
Pro Ile Val Asn Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
          20                  25                  30

TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC  230
Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
          35                  40                  45

TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA  275
Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
          50                  55                  60

GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC  320
Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
          65                  70                  75

TTC TCC AAC TGACAACTGG ATCC  343
Phe Ser Asn
          80
```

SEQ ID NO: 33

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM765)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG    50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG    95
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
            -10                                 -1   1

CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA   140
Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
          5                   10                  15

CCA ATA GTC AGC GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA   185
Pro Ile Val Ser Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
         20                  25                  30

TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC   230
Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
         35                  40                  45

TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA   275
Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
         50                  55                  60

GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC   320
Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
         65                  70                  75

TTC TCC AAC TGACAACTGG ATCC  343
Phe Ser Asn
         80
```

SEQ ID NO: 34

SEQUENCE LENGTH: 343

SEQUENCE TYPE: nucleic acid

MOLECULE TYPE: other nucleic acid

ORIGINAL SOURCE ORGANISM: *E. coli*

STRAIN: JE5505 (pM765)

SEQUENCE

```
AAGCTTAAAA AAGGGTATAA AATAAA ATG AAA CAA AGT ACT ATT GCA CTG   50
                              Met Lys Gln Ser Thr Ile Ala Leu
                                  -20                 -15

 GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC GCT GTG   95
 Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Ala Val
             -10                                 -1   1

 CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC TGT AAT CTA  140
 Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala Cys Asn Leu
           5                   10                  15

 CCA ATA GTC GCG GGC CCC TGC CGA GCC TTC ATC AAG CTC TGG GCA  185
 Pro Ile Val Ala Gly Pro Cys Arg Ala Phe Ile Lys Leu Trp Ala
          20                  25                  30

 TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG GGC  230
 Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly
          35                  40                  45

 TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG GAG TGC AGA  275
 Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys Glu Cys Arg
          50                  55                  60

 GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC  320
 Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg
          65                  70                  75

 TTC TCC AAC TGACAACTGG ATCC  343
 Phe Ser Asn
          80
```

Claims

1. A polypeptide which contains, at least as a part thereof, an amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the following amino acid sequence represented by formula 1;

```
                    5                   10
Cys Asn Leu Pro Ile Val Arg Gly Pro Cys

                   15                   20
Arg Ala Phe Ile Gln Leu Trp Ala Phe Asp

                   25                   30
Ala Val Lys Gly Lys Cys Val Leu Phe Pro

                   35                   40
Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys

                   45                   50
Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr

Cys                                     -- 1.
```

2. The polypeptide according to claim 1 wherein said amino acid substitution in the amino acid sequence of formula 1 is at least one substitution selected from the following substitutions (i) to (iii);
   (i) substitution of 15 position Gln counting from the N-terminus by an amino acid other than Gln,
   (ii) substitution of 42 position Tyr counting from the N-terminus by an amino acid other than Tyr, and
   (iii) substitution of 7 position Arg counting from the N-terminus by an amino acid other than Arg.

3. The polypeptide according to claim 1 or 2 wherein said amino acid substitution in the amino acid sequence of formula 1 is effective for improving at least one property of the polypeptide selected from the group consisting of;
   (i) increasing the activities of the polypeptide to inhibit FXa,
   (ii) having an improved secretion from a transformant when the polypeptide is produced by recombinant DNA techniques, and
   (iii) increasing the activities of the polypeptide to inhibit elastase.

4. The polypeptide according to any one of the claims 1 to 3 wherein said amino acid substitution in the amino acid sequence of formula 1 is at least one substitution selected from the following substitutions (1) to (11);
   (1) substitution of Lys for the 15 position Gln counting from the N-terminus,
   (2) substitution of Arg for the 15 position Gln counting from the N-terminus,
   (3) substitution of Glu for the 42 position Tyr counting from the N-terminus,
   (4) substitution of Asp for the 42 position Tyr counting from the N-terminus,
   (5) substitution of Glu for the 7 position Arg counting from the N-terminus,
   (6) substitution of Gln for the 7 position Arg counting from the N-terminus,
   (7) substitution of Asp for the 7 position Arg counting from the N-terminus, and
   (8) substitution of Leu for the 7 position Arg counting from the N-terminus,
   (9) substitution of Asn for the 7 position Arg counting from the N-terminus,
   (10) substitution of Ser for the 7 position Arg counting from the N-terminus, and
   (11) substitution of Ala for the 7 position Arg counting from the N-terminus.

5. The polypeptide according to any one of the claims 1 to 4 wherein said polypeptide contains, at least as a part thereof, an amino acid sequence in which the N-terminus of said amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the amino acid sequence of formula 1 is further supplemented with a second amino acid sequence selected from the following amino acid sequences (1) to (5);

(1) Asp Asp Ala Ala,

(2) Thr Val Ala Ala,

(3) Val Ala Ala,

(4) Ala Ala, and

(5) Ala.

6. The polypeptide according to any one of the claims 1 to 5 wherein said polypeptide contains, at least as a part thereof, an amino acid sequence in which the C-terminus of said amino acid sequence resulting from substitution of an amino acid for at least one amino acid in the amino acid sequence of formula 1 is further supplemented with a second amino acid sequence selected from the following amino acid sequences (1) to (15);

(1) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn,

(2) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe Ser,

(3) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe,

(4) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg,

(5) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu,

(6) Gly Val Pro Gly Asp Gly Asp Glu Glu Leu,

(7) Gly Val Pro Gly Asp Gly Asp Glu Glu,

(8) Gly Val Pro Gly Asp Gly Asp Glu,

(9) Gly Val Pro Gly Asp Gly Asp,

(10) Gly Val Pro Gly Asp Gly,

(11) Gly Val Pro Gly Asp,

(12) Gly Val Pro Gly,

(13) Gly Val Pro,

(14) Gly Val, and

(15) Gly.

7. The polypeptide according to any one of the claims 1 to 6 wherein said polypeptide has at least one activity to inhibit a protease.

8. A DNA fragment which comprises a nucleotide sequence coding for the polypeptide of any one of the claims 1 to 7.

9. The DNA fragment according to claim 8 wherein said fragment comprises, at least as a part thereof, a nucleotide sequence resulting from substitution of a nucleotide for at least one nucleotide in the

following nucleotide sequence represented by formula 2;

```
1           10            20            30
TGC AAT CTC CCC ATA GTC CGG GGC CCC TGC

            40            50            60
CGA GCC TTC ATC CAG CTC TGG GCA TTT GAT

            70            80            90
GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC

           100           110           120
TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG

           130           140           150
TTC TAC TCA GAG AAG GAG TGC AGA GAG TAC


TGC                                        -- 2.
```

10. The DNA fragment according to claim 8 or 9 wherein said nucleotide substitution in the nucleotide sequence of formula 2 is at least one substitution selected from the following substitutions (i) to (iii);
(i) substitution of at least one nucleotide for the 43 to 45 position nucleotide sequence CAG counting from the 5' end,
(ii) substitution of at least one nucleotide for the 124 to 126 position nucleotide sequence TAC counting from the 5' end, and
(iii) substitution of at least one nucleotide for the 19 to 21 position nucleotide sequence CGG counting from the 5' end.

11. The DNA fragment according to any one of the claims 8 to 10 wherein said nucleotide substitution in the nucleotide sequence of formula 2 is at least one substitution selected from the following substitutions (1) to (11);
(1) substitution of the 43 to 45 position nucleotide sequence CAG counting from the 5' end by other nucleotide sequence coding for Lys,
(2) substitution of the 43 to 45 position nucleotide sequence CAG counting from the 5' end by other nucleotide sequence coding for Gln,
(3) substitution of the 124 to 126 position nucleotide sequence TAC counting from the 5' end by other nucleotide sequence coding for Glu,
(4) substitution of the 124 to 126 position nucleotide sequence TAC counting from the 5' end by other nucleotide sequence coding for Asp,
(5) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Glu,
(6) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Gln,
(7) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Asp,
(8) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Leu,
(9) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Asn,
(10) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Ser, and
(11) substitution of the 19 to 21 position nucleotide sequence CGG counting from the 5' end by other nucleotide sequence coding for Ala.

12. The DNA fragment according to any one of the claims 8 to 11 wherein said nucleotide substitution in the nucleotide sequence of formula 2 is at least one substitution selected from the following substitutions (1) to (11);

(1) substitution of AAG for the 43 to 45 position nucleotide sequence CAG counting from the 5' end,
(2) substitution of CGT for the 43 to 45 position nucleotide sequence CAG counting from the 5' end,
(3) substitution of GAA for the 124 to 126 position nucleotide sequence TAC counting from the 5' end,
(4) substitution of GAC for the 124 to 126 position nucleotide sequence TAC counting from the 5' end,
(5) substitution of GAA for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(6) substitution of CAG for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(7) substitution of GAT for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(8) substitution of CTG for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(9) substitution of AAC for the 19 to 21 position nucleotide sequence CGG counting from the 5' end,
(10) substitution of AGC for the 19 to 21 position nucleotide sequence CGG counting from the 5' end, and
(11) substitution of GCG for the 19 to 21 position nucleotide sequence CGG counting from the 5' end.

13. The DNA fragment according to any one of the claims 8 to 12 wherein said fragment contains, at least as a part thereof, a nucleotide sequence in which the 5' end of said nucleotide sequence resulting from substitution of a nucleotide for at least one nucleotide in the nucleotide sequence of formula 2 is further supplemented with a second nucleotide sequence selected from the following nucleotide sequences (1) to (5);

(1) GAC GAC GCC GCC,

(2) ACC GTC GCC GCC,

(3) GTC GCC GCC,

(4) GCC GCC, and

(5) GCC.

14. The DNA fragment according to any one of the claims 8 to 13 wherein said fragment contains, at least as a part thereof, a nucleotide sequence in which the 3' end of said nucleotide sequence resulting from substitution of a nucleotide for at least one nucleotide in the nucleotide sequence of formula 2 is further supplemented with a second nucleotide sequence selected from the following nucleotide sequences (1) to (15);

(1) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC TCC AAC,

(2) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC TCC,

(3) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC,

(4) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC,

(5) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG,

(6) GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG,

(7) GGT GTC CCT GGT GAT GGT GAT GAG GAG,

(8) GGT GTC CCT GGT GAT GGT GAT GAG,

(9) GGT GTC CCT GGT GAT GGT GAT,

(10) GGT GTC CCT GGT GAT GGT,

(11) GGT GTC CCT GGT GAT,

(12) GGT GTC CCT GGT,

(13) GGT GTC CCT,

(14) GGT GTC, and

(15) GGT.

15. A vector which contains the DNA fragment of any one of the claims 8 to 14.

16. A transformant transformed with the DNA fragment of any one of the claims 8 to 14.

17. A transformant transformed with the vector of claim 15.

18. A process for producing the polypeptide of any one of the claims 1 to 7, which comprises the steps of:
a) preparing a DNA fragment containing a nucleotide sequence which encodes the polypeptide of any one of the claims 1 to 7,
b) preparing a transformant by transforming a host cell with the DNA fragment obtained in the above step a), and
c) culturing the transformant obtained in the above step b) to allow the transformant to produce the polypeptide of any one of the claims 1 to 7 and subsequently recovering said polypeptide from the culture medium.

19. A process for producing the polypeptide of any one of the claims 1 to 7, which comprises the steps of:
a) preparing a DNA fragment containing a nucleotide sequence which encodes the polypeptide of any one of the claims 1 to 7,
b) preparing a vector which contains the DNA fragment obtained in the above step a),
c) preparing a transformant by transforming a host cell with the vector obtained in the above step b), and
d) culturing the transformant obtained in the above step c) to allow the transformant to produce the polypeptide of any one of the claims 1 to 7 and subsequently recovering said polypeptide from the culture medium.

20. A drug composition which contains the polypeptide of any one of the claims 1 to 7 as an active ingredient.

21. The drug composition according to claim 20 wherein said composition is used for the prevention and/or treatment of at least one disease which is selected from the group consisting of infestation, multiple

organ failure, shock, pancreatitis, disseminated intravascular coagulation syndrome, ischemic heart disease, nephritis, hepatic cirrhosis, re-obstruction at the time of blood circulation reconstructive operation, edema caused by increased vascular permeability, adult respiratory distress syndrome, rheumatoid arthritis, arthritis and allergic diseases.

22. An protease inhibition process which comprises using the polypeptide of any one of the claims 1 to 7.

Fig. 1

S 3 3

5' AGCTTAAAAA AGGGTATAAA ATAAAATGAA AC

ATTTTT TCCCATATTT TATTTTACTT TGTTTCATGA 5'

S 3 4

S 3 5

5' AAAGTACTAT TGCACTGGCA CTCTTACCGT TACTGTTT

TA ACGTGACCGT GAGAATGGCA ATGACAAA TGGGGA 5'

S 1 8

S 1 9

5' ACCCCTGTGA CAAAAGCCGA CTCCCTAGGT CG

CACT GTTTTCGGCT GAGGGATCCA GC 5'

S 2 0

Fig. 2

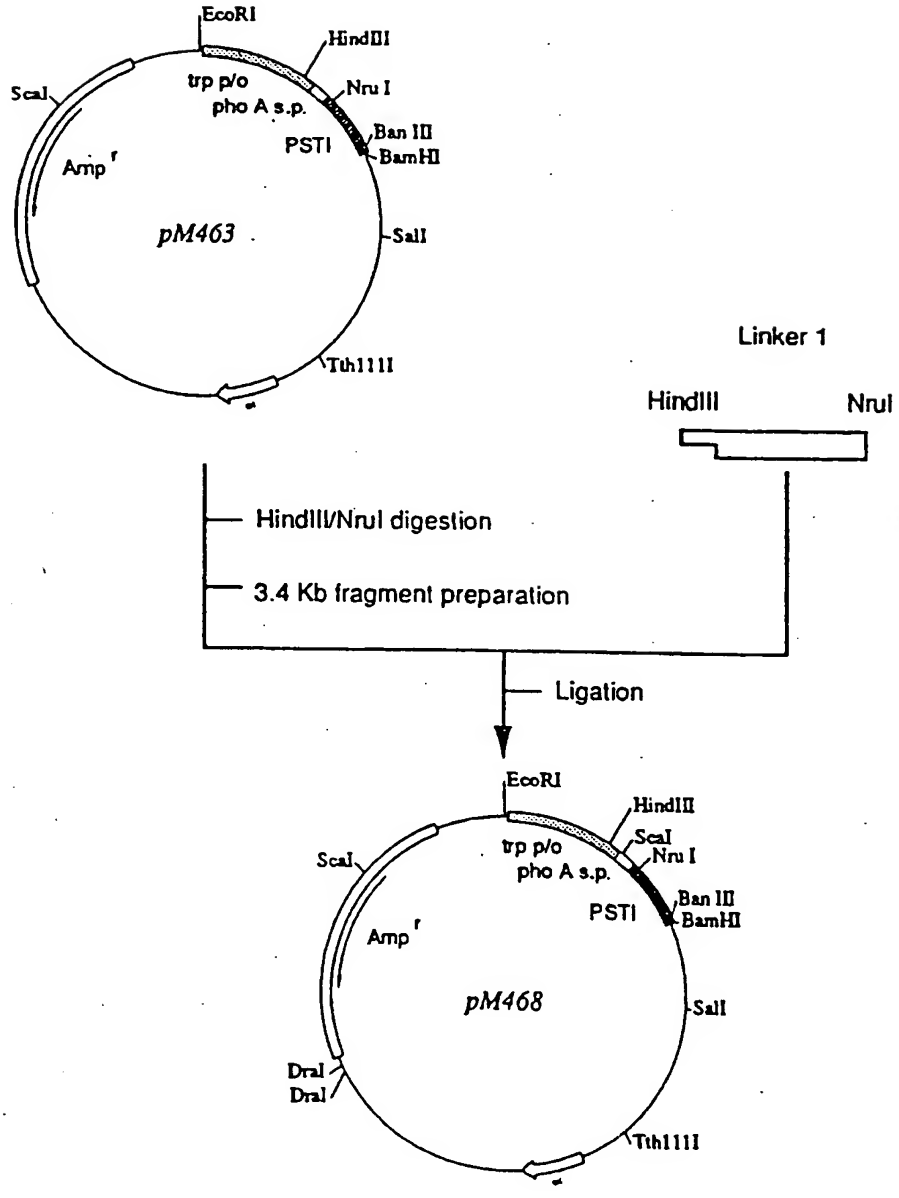

EcoRI
HindIII
trp p/o
pho A s.p.
Nru I
ScaI
Ban III
PSTI
BamHI
Amp r
pM463
SalI
Tth111I
Linker 1
HindIII
NruI
HindIII/NruI digestion
3.4 Kb fragment preparation
Ligation
EcoRI
HindIII
ScaI
trp p/o
pho A s.p.
Nru I
ScaI
Ban III
PSTI
BamHI
Amp r
pM468
SalI
DraI
DraI
Tth111I

Fig. 3

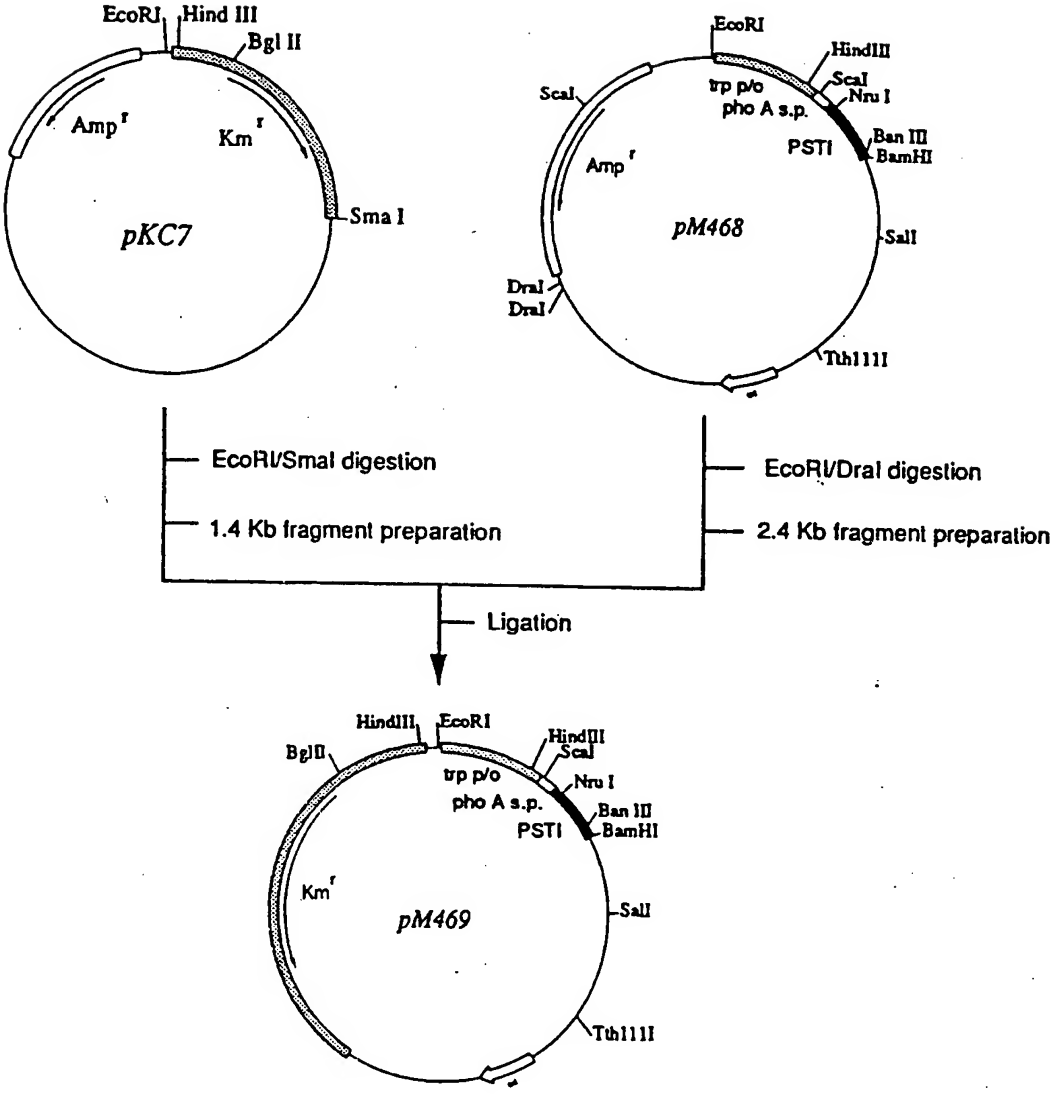


Fig. 4

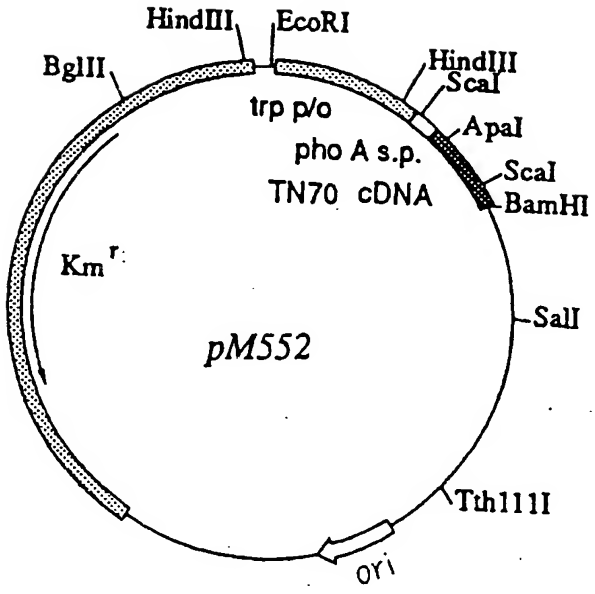


Fig. 5

Y46E primer 5' GG AAC AAG TTC GAA TCA GAG AAG GA 3'

Fig. 6

ScaI sense primer 5' ACT ATT GCA CTG GCA CTC TTA 3'

Fig. 7

BamHI primer 5' TGGATCCAG TTG TCA GTT GGA GAA GC 3'

DOCID: <EP__0543240A1_I_>

Fig. 8

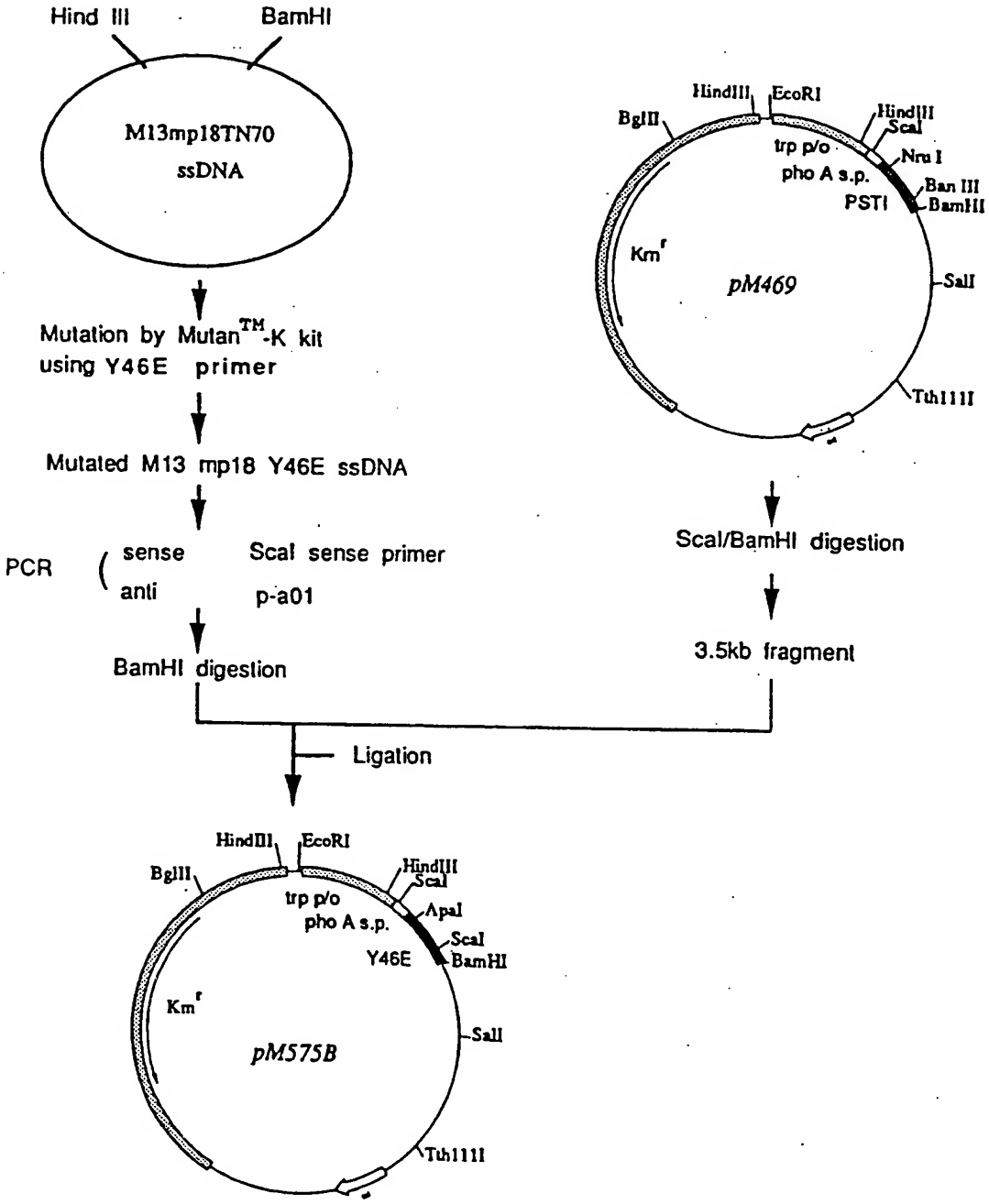
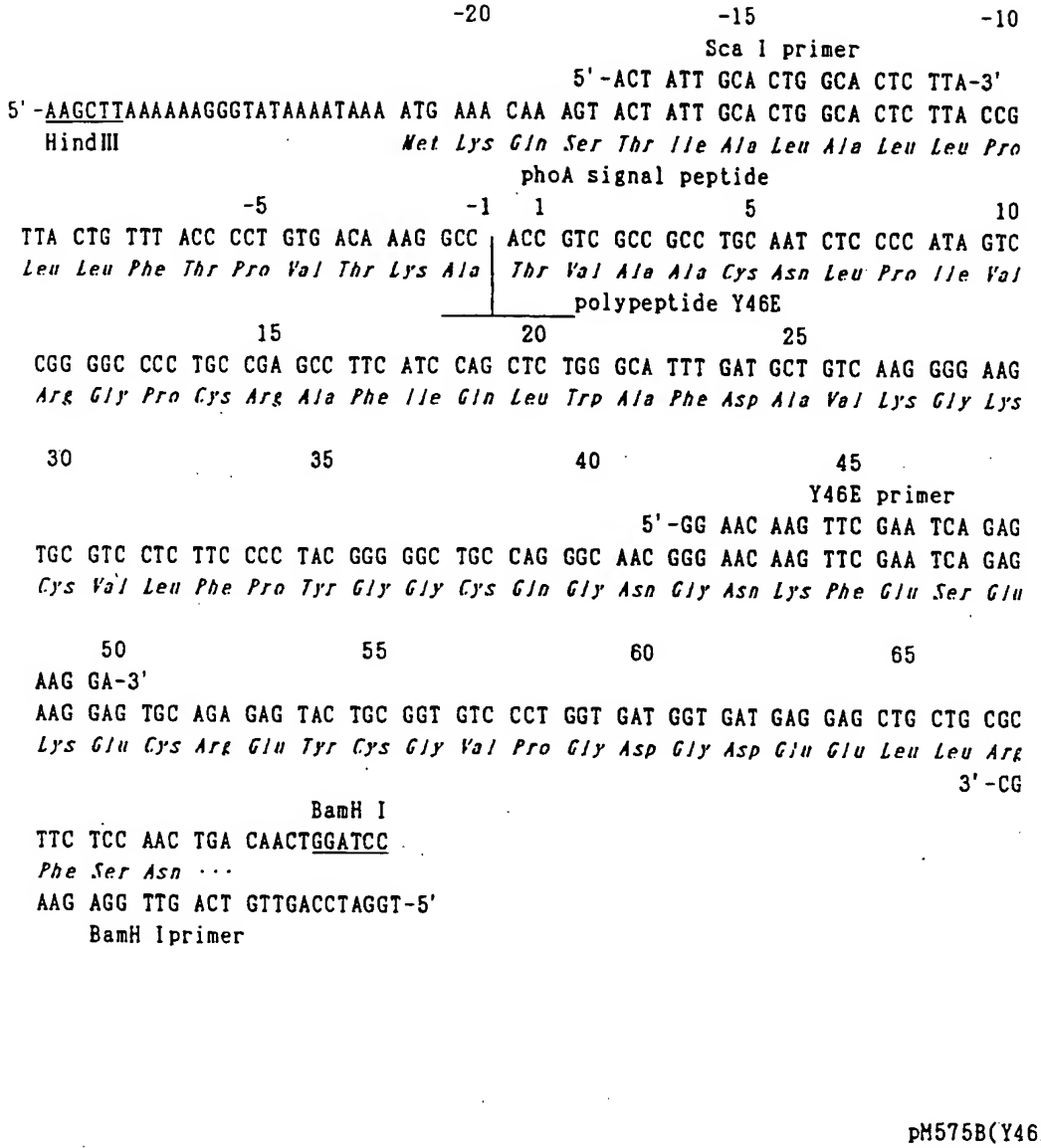


Fig. 9



pM575B(Y46E)

Fig. 10

Oligomer TV12DD 5' TG ACA AAG GCC GAC GAC GCC GCC TGC AA 3'

Fig. 11

HindIII primer 5' ACGCAAGTTCACGTAAAAAGC 3'

Fig. 12

Q19K primer 5' C AAA TGC CCA GAG CTT GAT GAA GGC TCG GCA 3'

Fig. 13

pBR BamHI primer 5' ACGATGCGTTCCGGCGTAGAG 3'

Fig. 14

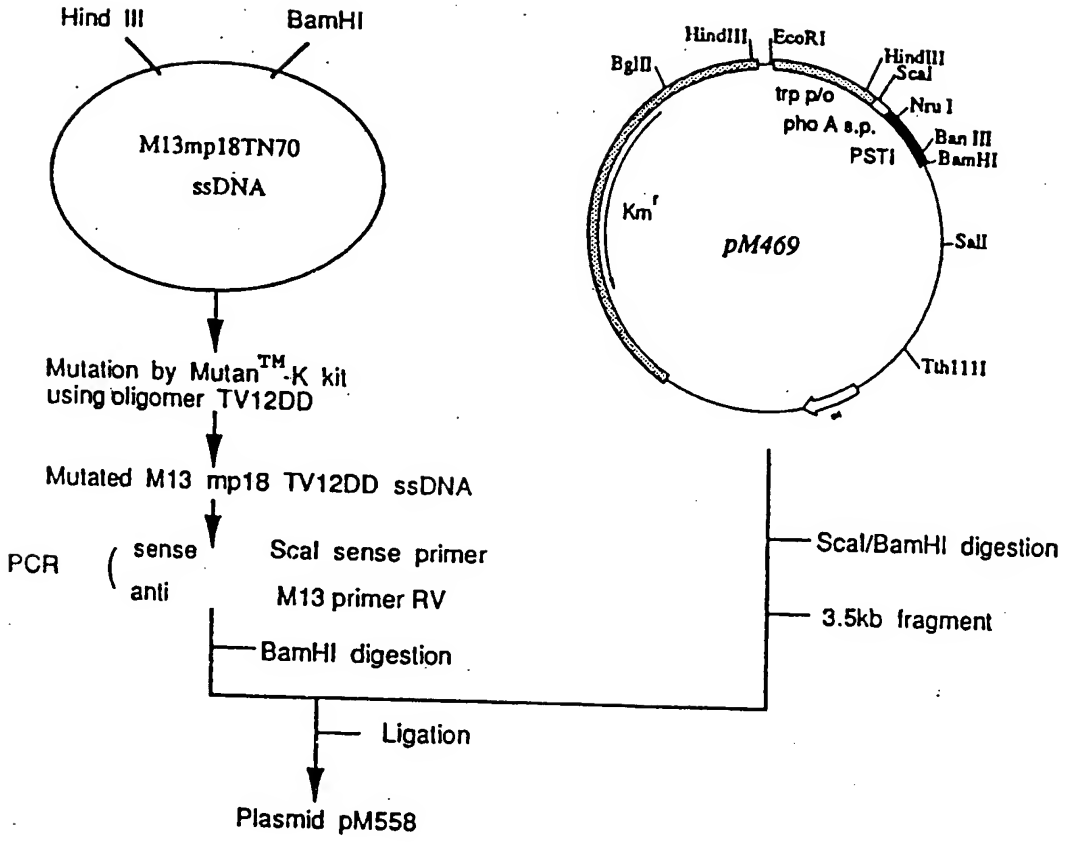

Hind III
BamHI
M13mp18TN70
ssDNA
Mutation by Mutan™-K kit
using oligomer TV12DD
Mutated M13 mp18 TV12DD ssDNA
PCR
sense
anti
ScaI sense primer
M13 primer RV
BamHI digestion
Ligation
Plasmid pM558
HindIII
EcoRI
BglII
HindIII
ScaI
trp p/o
pho A s.p.
Nru I
Ban III
PSTI
BamHI
Km^r
pM469
SalI
Tth111I
ScaI/BamHI digestion
3.5kb fragment

Fig. 15

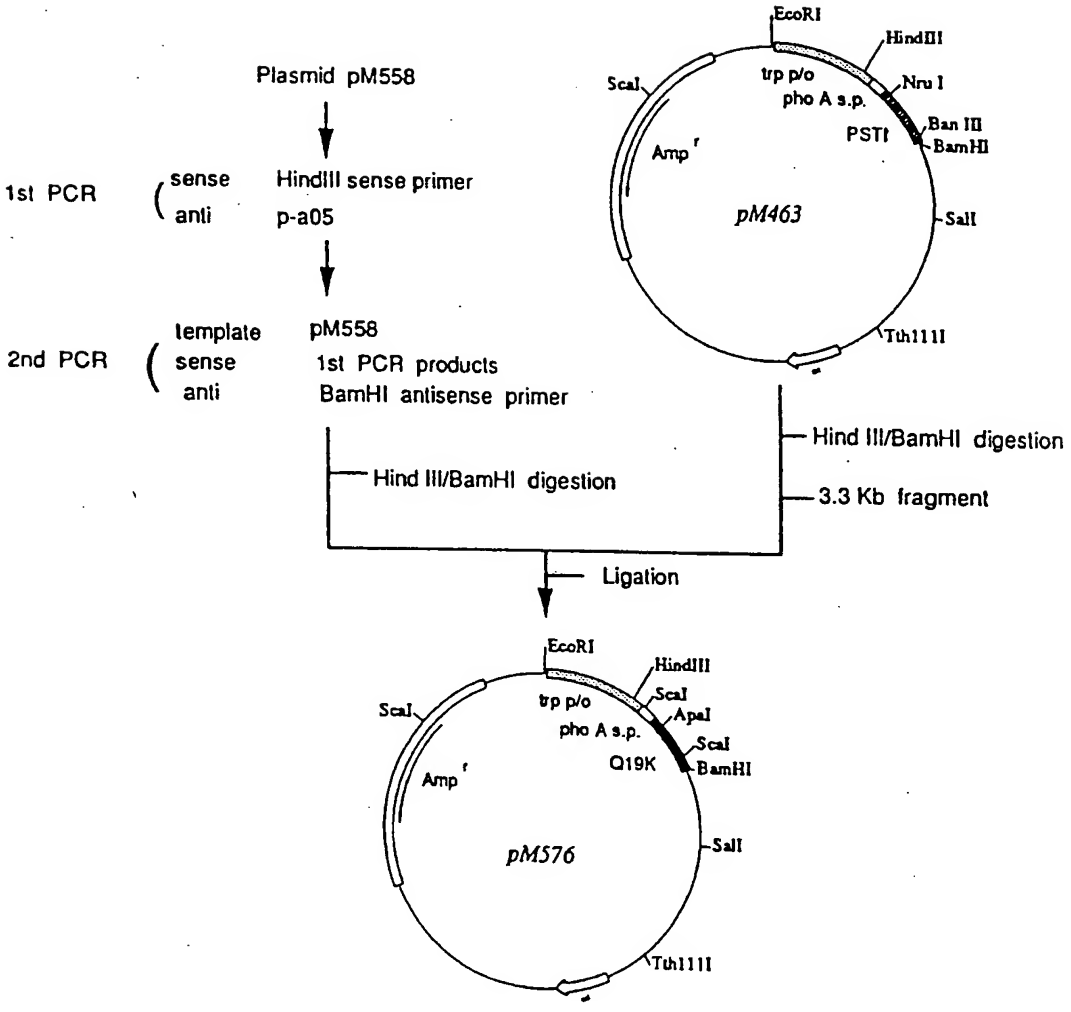
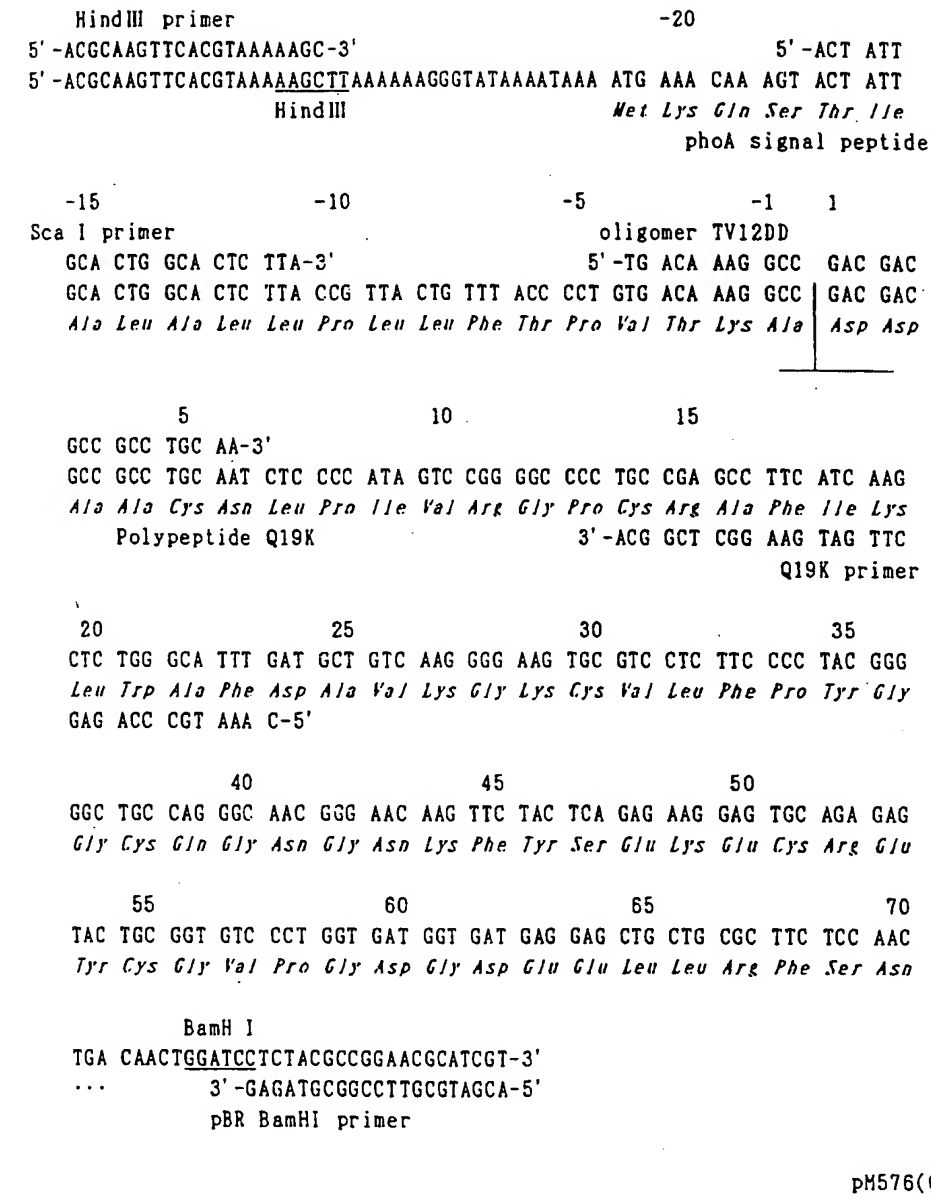

Plasmid pM558
1st PCR
sense
anti
HindIII sense primer
p-a05
2nd PCR
template
sense
anti
pM558
1st PCR products
BamHI antisense primer
Hind III/BamHI digestion
Hind III/BamHI digestion
3.3 Kb fragment
Ligation
EcoRI
HindIII
ScaI
trp p/o
pho A s.p.
Nru I
Ban III
PSTI
BamHI
Amp r
pM463
SalI
Tth111I
EcoRI
HindIII
ScaI
trp p/o
pho A s.p.
ScaI
ApaI
ScaI
Q19K
BamHI
Amp r
pM576
SalI
Tth111I

Fig. 16



pM576(Q19K)

Fig. 17

AN68 primer 5'-CTATTGG TAG ATT ACA GGC CGC GGC CTT TGT CAC AGG GGT-3'

Fig. 18

SacII primer 5'-AAG GCC GCG GCC TGT AAT CTA CCA ATA GTC-3'

Fig. 19

Q19R primer 5'-ATC AAA TGC CCA GAG ACG GAT GAA GGC TCG GC-3'

Fig. 20

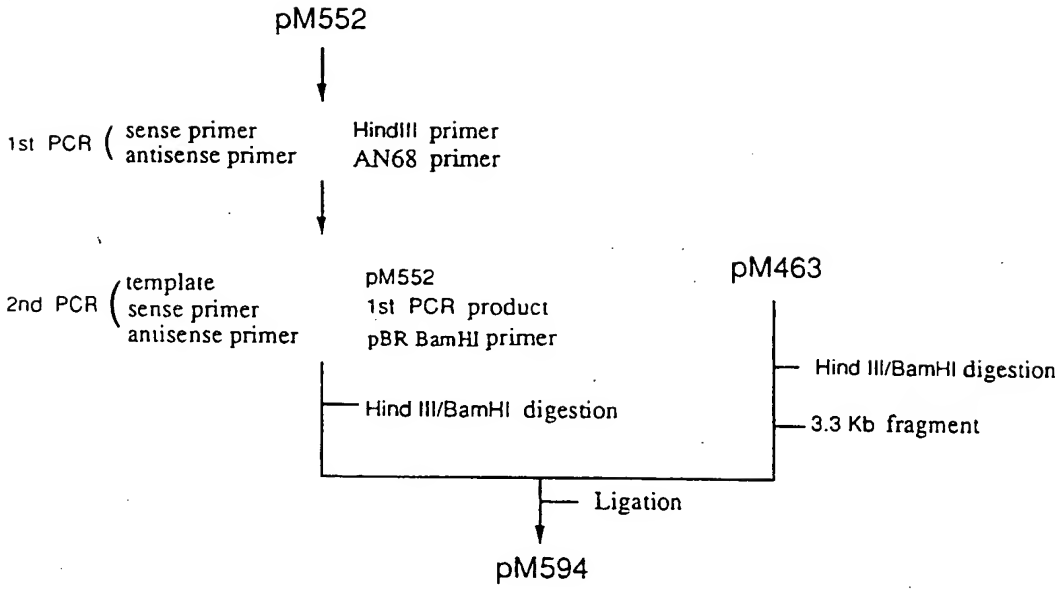


Fig. 21

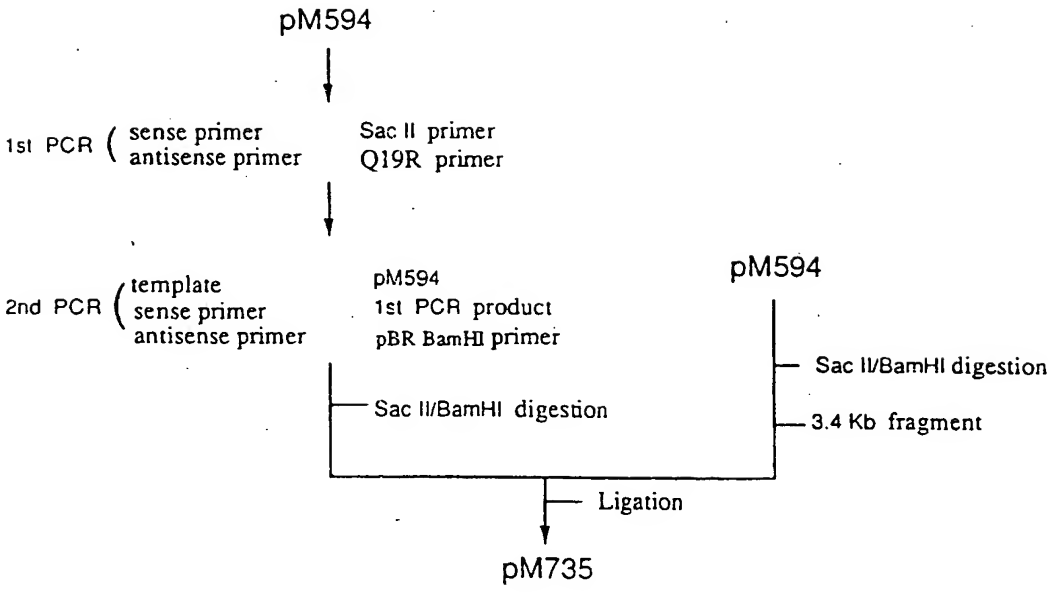

pM594
1st PCR (sense primer
antisense primer
Sac II primer
Q19R primer
2nd PCR (template
sense primer
antisense primer
pM594
1st PCR product
pBR BamHI primer
pM594
Sac II/BamHI digestion
3.4 Kb fragment
Sac II/BamHI digestion
Ligation
pM735

Fig. 22

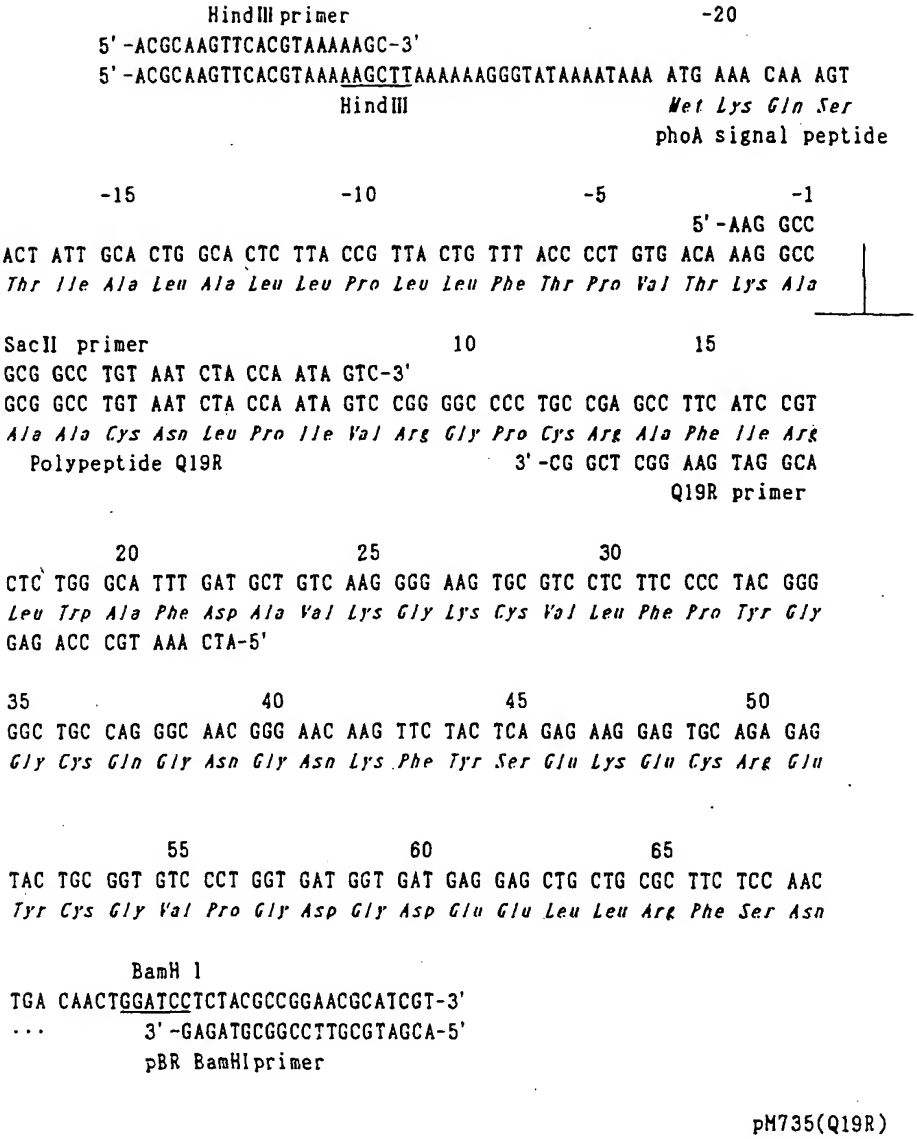


Fig. 23

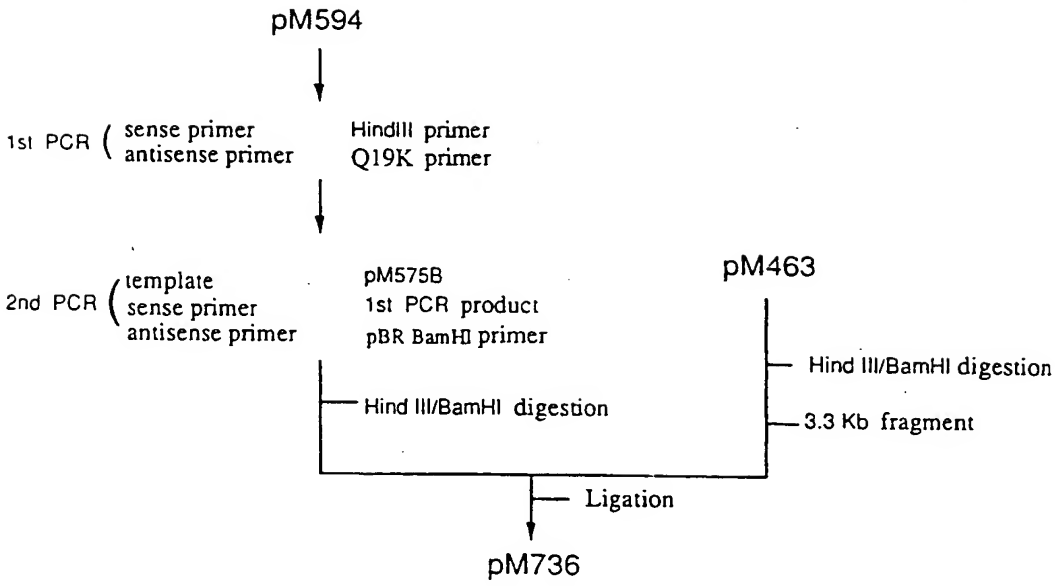

pM594
1st PCR
sense primer
antisense primer
HindIII primer
Q19K primer
2nd PCR
template
sense primer
antisense primer
pM575B
1st PCR product
pBR BamHI primer
Hind III/BamHI digestion
pM463
Hind III/BamHI digestion
3.3 Kb fragment
Ligation
pM736

Fig. 24

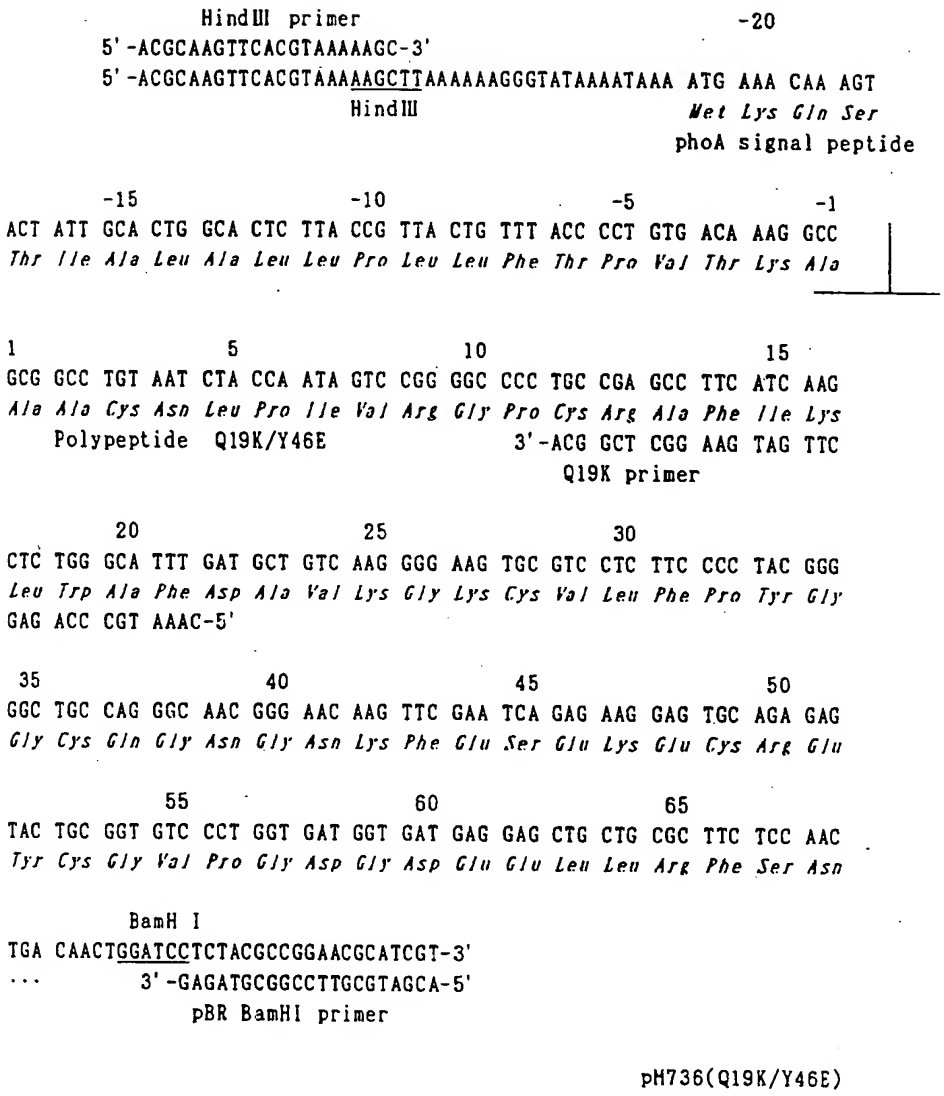

HindIII primer
-20
5'-ACGCAAGTTCACGTAAAAAGC-3'
5'-ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT
HindIII
Met Lys Gln Ser
phoA signal peptide
-15 -10 -5 -1
ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG GCC
Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala
1 5 10 15
GCG GCC TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC AAG
Ala Ala Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Lys
Polypeptide Q19K/Y46E
3'-ACG GCT CGG AAG TAG TTC
Q19K primer
20 25 30
CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC TAC GGG
Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly
GAG ACC CGT AAAC-5'
35 40 45 50
GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG GAG TGC AGA GAG
Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys Glu Cys Arg Glu
55 60 65
TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG CTG CTG CGC TTC TCC AAC
Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu Arg Phe Ser Asn
BamH I
TGA CAACTGGATCCTCTACGCCGGAACGCATCGT-3'
... 3'-GAGATGCGGCCTTGCGTAGCA-5'
pBR BamHI primer
pH736(Q19K/Y46E)

Fig. 25

R11E primer 5'-TCG GCA GGG GCC TTC GAC TAT TGG TAG-3'

Fig. 26

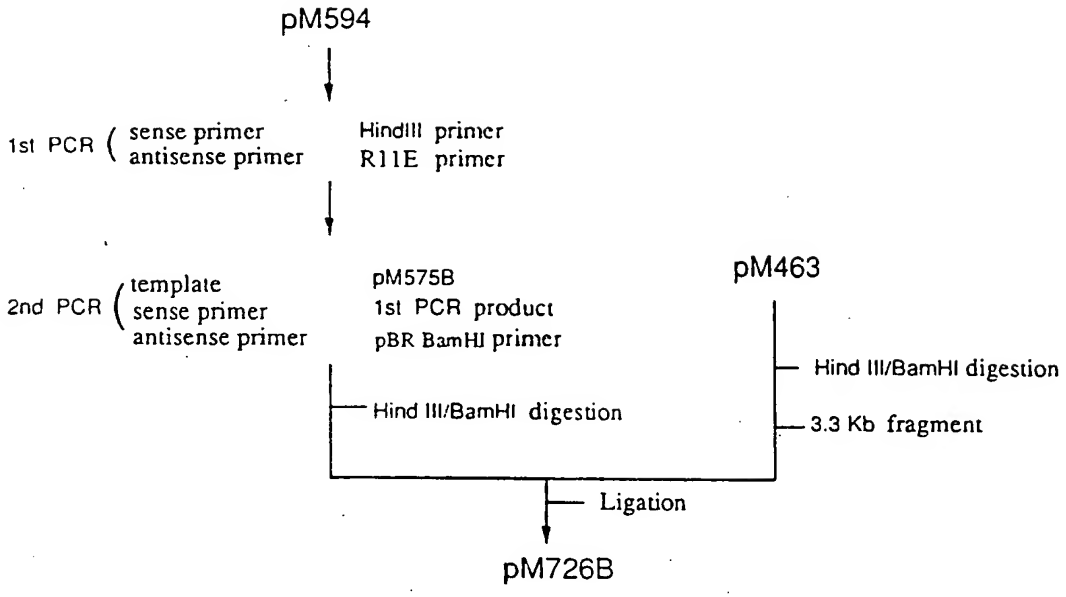
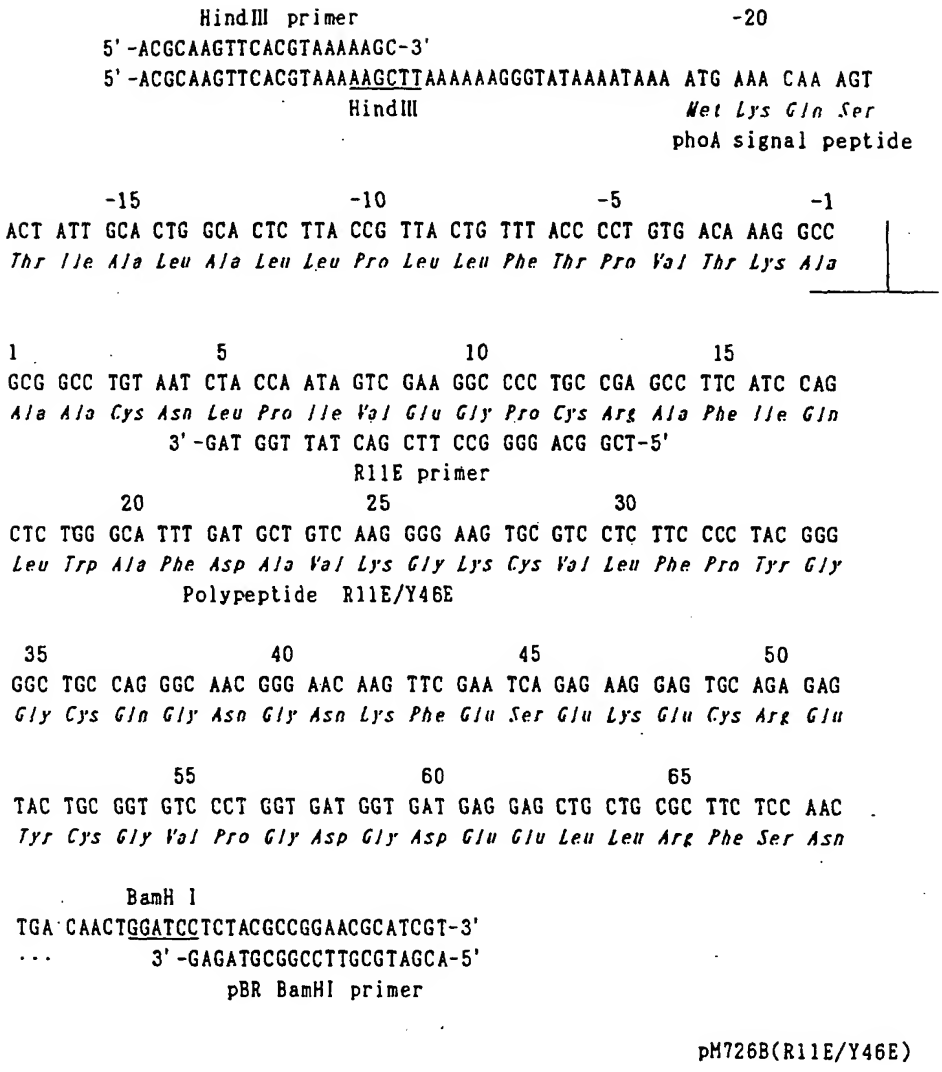


Fig. 27



pM726B(R11E/Y46E)

Fig. 28

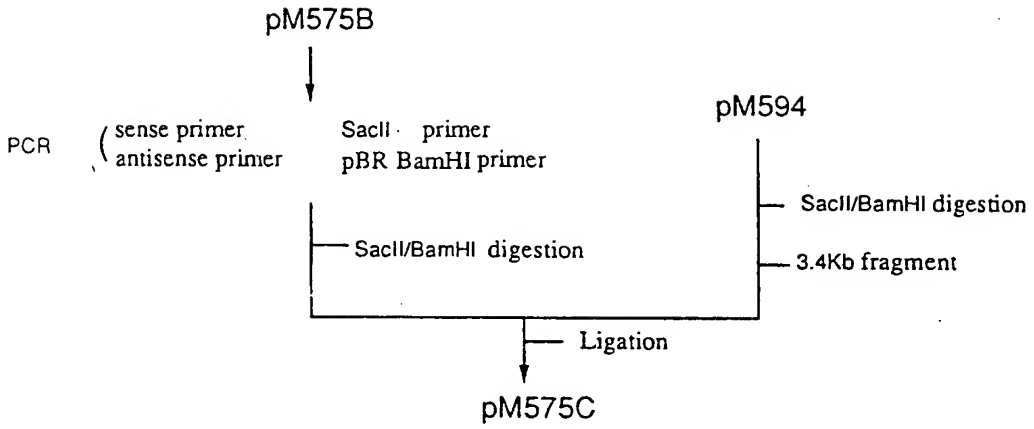
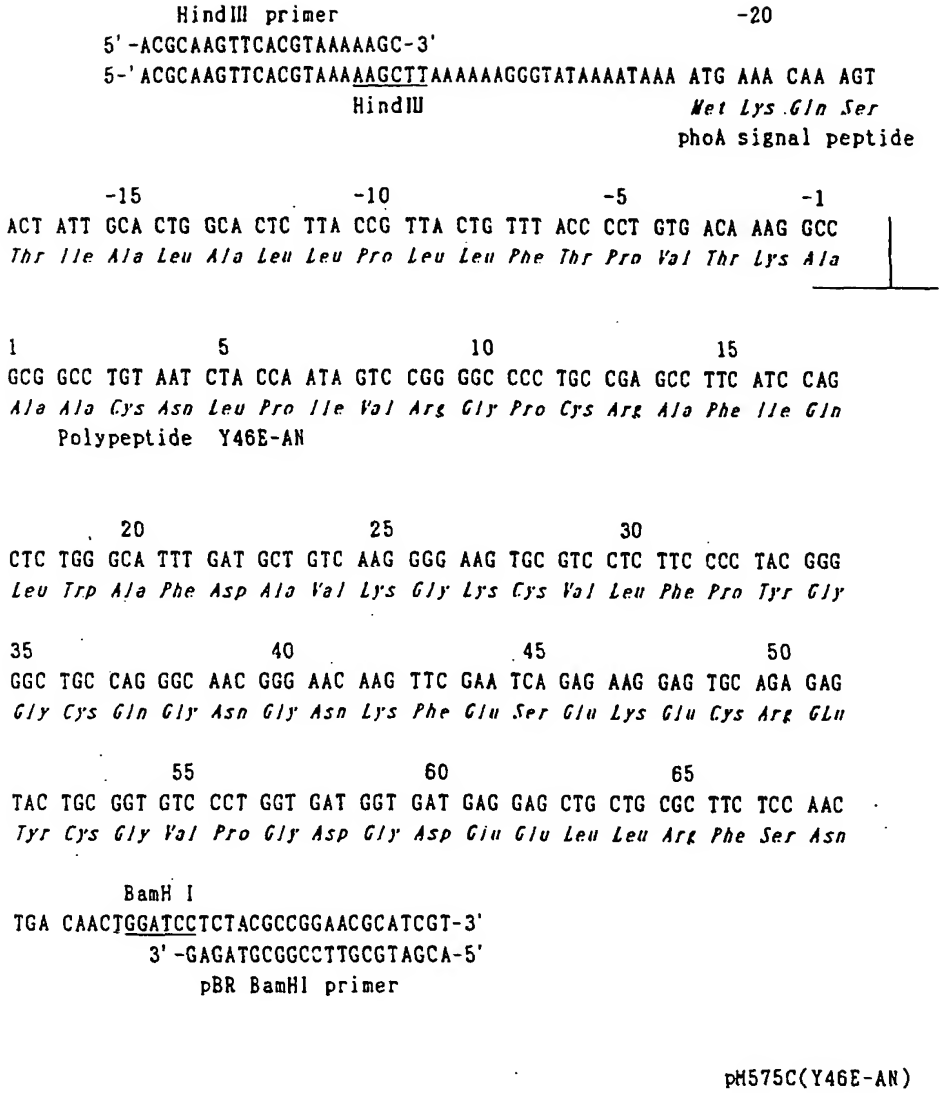

pM575B
PCR
sense primer
antisense primer
SacII primer
pBR BamHI primer
SacII/BamHI digestion
pM594
SacII/BamHI digestion
3.4Kb fragment
Ligation
pM575C

Fig. 29



pM575C(Y46E-AN)

Fig. 30

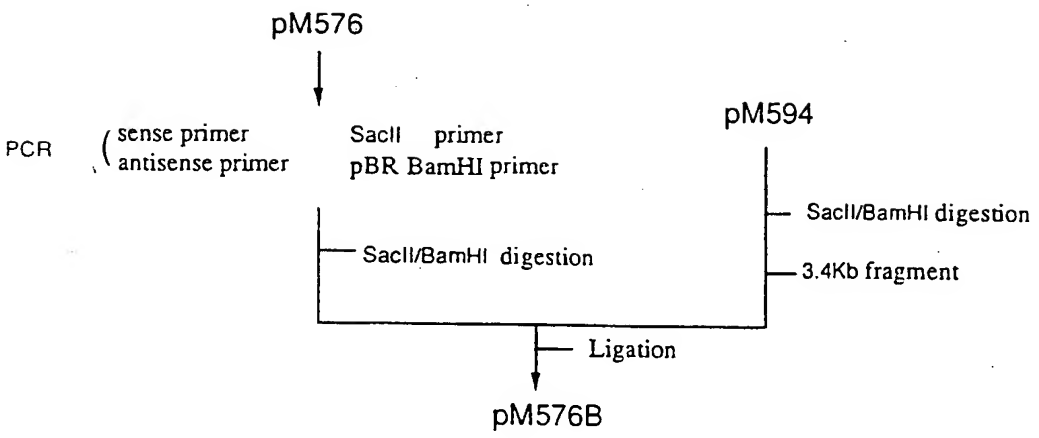
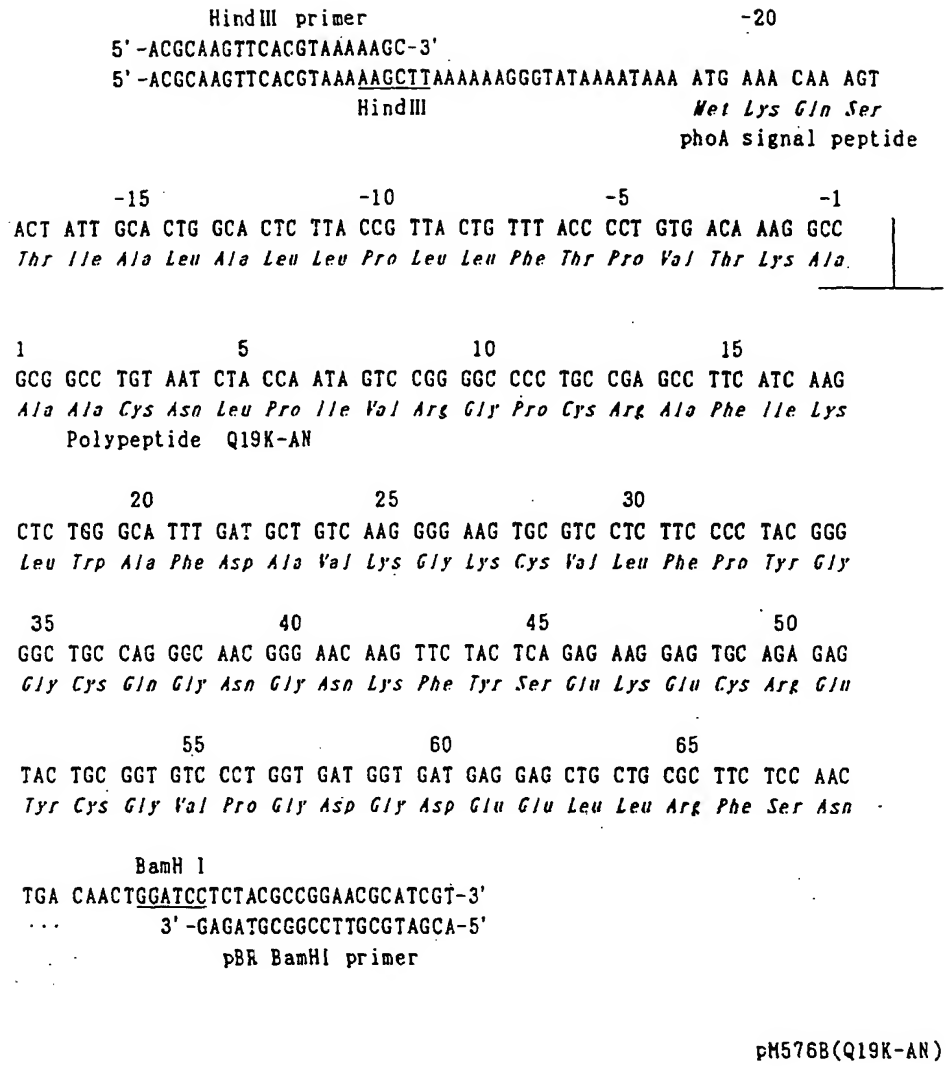

pM576
PCR
sense primer
antisense primer
SacII primer
pBR BamHI primer
SacII/BamHI digestion
pM594
SacII/BamHI digestion
3.4Kb fragment
Ligation
pM576B

Fig. 31



pM576B(Q19K-AN)

Fig. 32

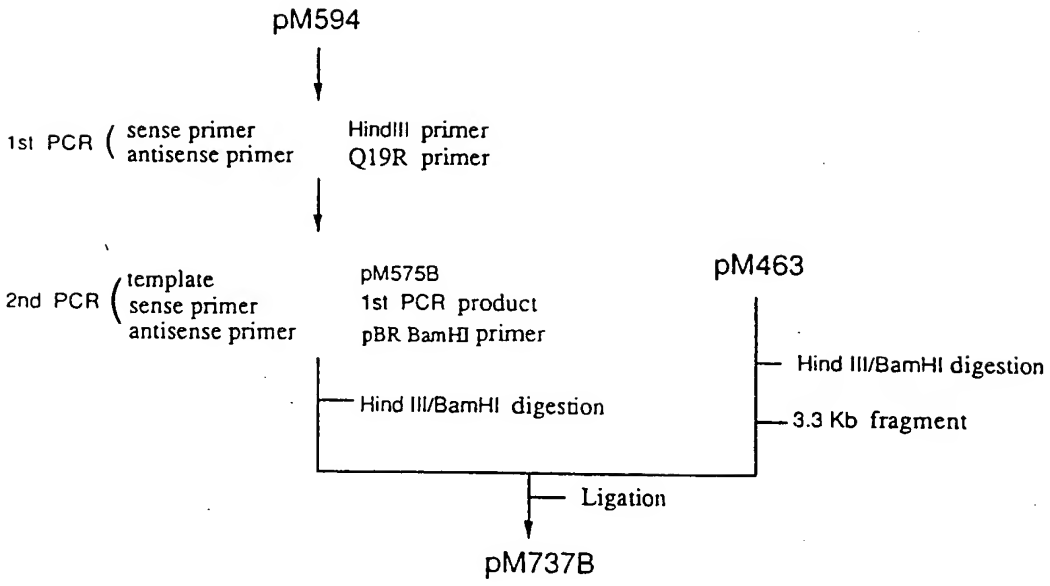
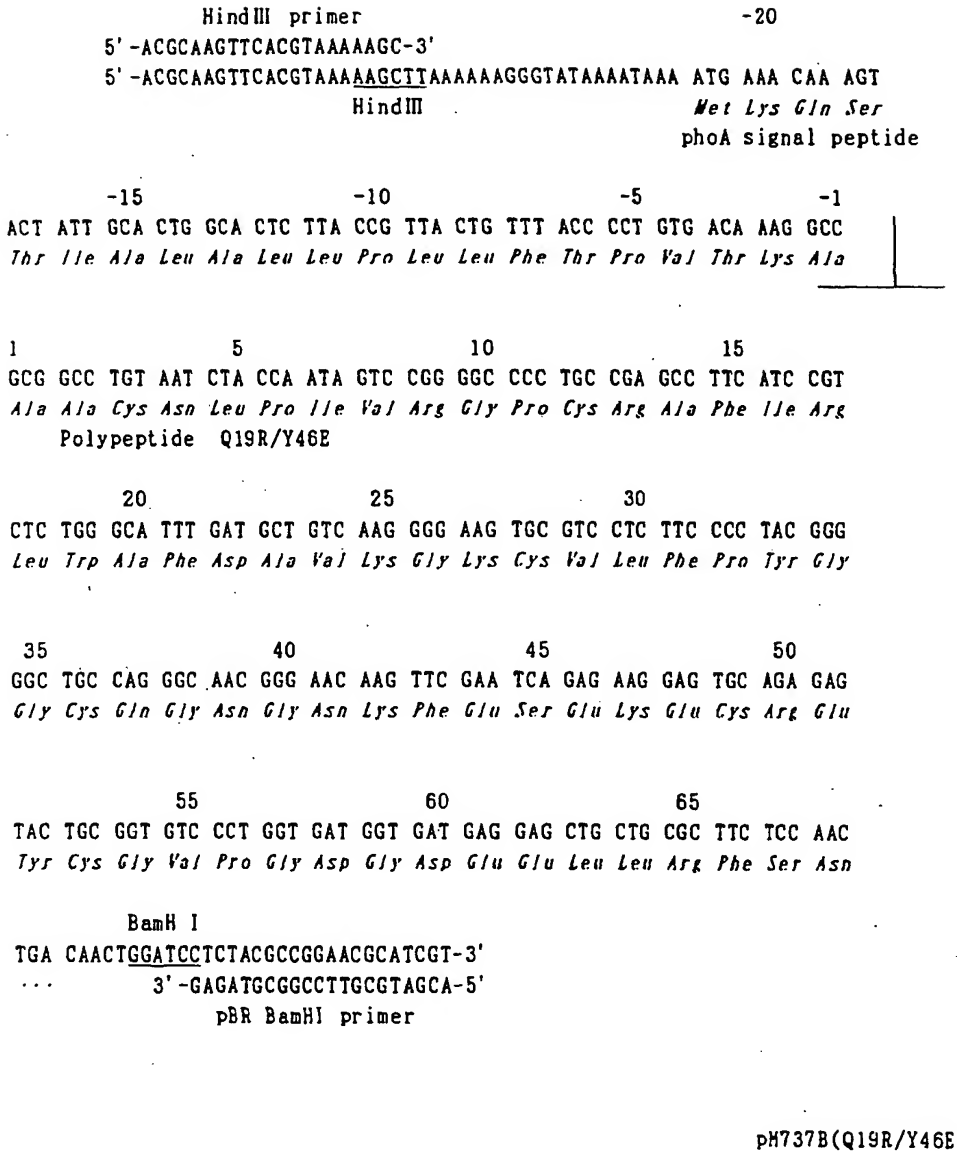

pM594
1st PCR
sense primer
antisense primer
HindIII primer
Q19R primer
2nd PCR
template
sense primer
antisense primer
pM575B
1st PCR product
pBR BamHI primer
pM463
Hind III/BamHI digestion
3.3 Kb fragment
Hind III/BamHI digestion
Ligation
pM737B

Fig. 33



pH737B(Q19R/Y46E)

Fig. 34

Linker 710

AGCTTAAAAA AGGGTATAAA ATAAAATGAA ACAAAGTACT ATTGCACTGG CACTCTTACC
ATTTTT TCCCATATTT TATTTTACTT TGTTTCATGA TAACGTGACC GTGAGAATGG

GTTACTGTTT ACCCCTGTGA CAAAGGCCGC TGTGCTACCG CAAGAAGAAG AAGGCTCGGG
CAATGACAAA TGGGGACACT GTTTCCGGCG ACACGATGGC GTTCTTCTTC TTCCGAGCCC

TATGGCCGCC TGTAATCTAC CAATAGTCCG GGGCC
ATACCGGCGG ACATTAGATG GTTATCAGGC C

Fig. 35

Y46D primer 5' GGG AAC AAG TTC GAC TCA GAG AAG G 3'

Fig. 36 JE5505(pM727)Q19K/Y46D

```
        HindIII  primer
5'-ACGCAAGTTCACGTAAAAAGC-3'
   ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT ACT
                  HindIII                      Met Lys Gln Ser Thr


      ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG
      Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys


      GCC GCT GTG CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC
      Ala Ala Val Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala
                                                              1

      TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC AAG
      Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Lys
                                    3'-ACG GCT CGG AAG TAG TTC
                                                       Q19K primer
               5                   10                  15

      CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC
      Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
      GAG ACC CGT-5'
               20                  25                  30

                                        Y46D primer
                              5'- GGG AAC AAG TTC GAC TCA GAG AAG
      TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG
      Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys
               35                  40                  45

      G-3'
      GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG
      Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
               50                  55                  60

                                        BamHI
      CTG CTG CGC TTC TCC AAC TGA CAACTGGATCCTCTACGCCGGACGCATCGT
      Leu Leu Arg Phe Ser Asn ...       3'- GAGATGCGGCCTGCGTAGCA-5'
               65          68                pBRBamHI primer
```

Fig. 37 JE5505(pM744)Q19R/Y46D

```
        HindIII  primer
5'-ACGCAAGTTCACGTAAAAAGC-3'
   ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT ACT
                  HindIII                    Met Lys Gln Ser Thr


      ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG
      Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys


      GCC GCT GTG CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC
      Ala Ala Val Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala
                                                             1

      TGT AAT CTA CCA ATA GTC CGG GGC CCC TGC CGA GCC TTC ATC CGT
      Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Arg
                                    3'-CG GCT CGG AAG TAG GCA
                                                  Q19R primer
               5                  10                  15

      CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC
      Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
      GAG ACC CGT AAA CTA-5'
               20                  25                  30

                                  Y46D primer
                           5'- GGG AAC AAG TTC GAC TCA GAG AAG
      TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG
      Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys
               35                  40                  45

      G-3'
      GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG
      Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
               50                  55                  60

                                        BamHI
      CTG CTG CGC TTC TCC AAC TGA CAACTGGATCCTCTACGCCGGACGCATCGT
      Leu Leu Arg Phe Ser Asn ...       3'- GAGATGCGGCCTGCGTAGCA-5'
               65          68                 pBRBamHI primer
```

Fig. 38

R11Q primer 5' CG GCA GGG GCC CTG GAC TAT TGG TA 3'

Fig. 39 R11Q/Q19K/Y46D JE5505(pM741)

```
      HindIII  primer
5'-ACGCAAGTTCACGTAAAAAGC-3'
   ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT ACT
                  HindIII                      Met Lys Gln Ser Thr


      ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG
      Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys


      GCC GCT GTG CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC
      Ala Ala Val Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala
                                                              1

      TGT AAT CTA CCA ATA GTC CAG GGC CCC TGC CGA GCC TTC ATC AAG
      Cys Asn Leu Pro Ile Val Gln Gly Pro Cys Arg Ala Phe Ile Lys
            3'-AT GGT TAT CAG GTC CCG GGG ACG GC-5'
                 R11Q primer           3'-ACG GCT CGG AAG TAG TTC
                                                    Q19K primer
               5                  10                  15

   \  CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC
      Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
      GAG ACC CGT-5'
               20                  25                  30

                                        Y46D primer
                              5'- GGG AAC AAG TTC GAC TCA GAG AAG
      TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG
      Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys
               35                  40                  45

      G-3'
      GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG
      Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
               50                  55                  60

                                        BamHI
      CTG CTG CGC TTC TCC AAC TGA CAACTGGATCCTCTACGCCGGACGCATCGT
      Leu Leu Arg Phe Ser Asn ...       3'- GAGATGCGGCCTGCGTAGCA-5'
               65          68                 pBRBamHI primer
```

Fig. 40

R11D primer 5' CG GCA GGG GCC ATC GAC TAT TGG TA 3'

Fig. 41 R11D/Q19K/Y46D JE5505 (pM742)

```
       HindIII  primer
5'-ACGCAAGTTCACGTAAAAAGC-3'
   ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT ACT
                  HindIII                      Met Lys Gln Ser Thr


      ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG
      Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys


      GCC GCT GTG CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC
      Ala Ala Val Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala
                                                             1

      TGT AAT CTA CCA ATA GTC GAT GGC CCC TGC CGA GCC TTC ATC AAG
      Cys Asn Leu Pro Ile Val Asp Gly Pro Cys Arg Ala Phe Ile Lys
            3'-AT GGT TAT CAG CTA CCG GGG ACG GC-5'
                R11D primer             3'-ACG GCT CGG AAG TAG TTC
                                                      Q19K primer
                5                   10                  15

      CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC
      Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
      GAG ACC CGT-5'
               20                  25                  30

                                        Y46D primer
                              5'- GGG AAC AAG TTC GAC TCA GAG AAG
      TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG
      Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys
               35                  40                  45

      G-3'
      GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG
      Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
               50                  55                  60

                                        BamHI
      CTG CTG CGC TTC TCC AAC TGA CAACTGGATCCTCTACGCCGGACGCATCGT
      Leu Leu Arg Phe Ser Asn ...       3'- GAGATGCGGCCTGCGTAGCA-5'
               65          68                 pBRBamHI primer
```

Fig. 42

R11L primer 5' CG GCA GGG GCC CAG GAC TAT TGG TA 3'

Fig. 43 JE5505 (pM743) R11L/Q19K/Y46D

```
      HindIII  primer
5'-ACGCAAGTTCACGTAAAAAGC-3'
   ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT ACT
                  HindIII                      Met Lys Gln Ser Thr


     ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG
     Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys


     GCC GCT GTG CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC
     Ala Ala Val Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala
                                                            1

     TGT AAT CTA CCA ATA GTC CTG GGC CCC TGC CGA GCC TTC ATC AAG
     Cys Asn Leu Pro Ile Val Leu Gly Pro Cys Arg Ala Phe Ile Lys
           3'-AT GGT TAT CAG GAC CCG GGG ACG GC-5'
               R11L primer             3'-ACG GCT CGG AAG TAG TTC
                                                    Q19K primer
               5                  10                  15

     CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC
     Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
     GAG ACC CGT-5'
              20                  25                  30

                                      Y46D primer
                           5'- GGG AAC AAG TTC GAC TCA GAG AAG
     TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAC TCA GAG AAG
     Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Asp Ser Glu Lys
              35                  40                  45

     G-3'
     GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG
     Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
              50                  55                  60

                                      BamHI
     CTG CTG CGC TTC TCC AAC TGA CAACTGGATCCTCTACGCCGGACGCATCGT
     Leu Leu Arg Phe Ser Asn ...       3'- GAGATGCGGCCTGCGTAGCA-5'
              65          68                 pBRBamHI primer
```

Fig. 44 R11E/Q19K/Y46E

```
       HindIII  primer
5'-ACGCAAGTTCACGTAAAAAGC-3'
   ACGCAAGTTCACGTAAAAAGCTTAAAAAAGGGTATAAAATAAA ATG AAA CAA AGT ACT
                  HindIII                      Met Lys Gln Ser Thr


      ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAG
      Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys


      GCC GCT GTG CTA CCG CAA GAA GAA GAA GGC TCG GGT ATG GCC GCC
      Ala Ala Val Leu Pro Gln Glu Glu Glu Gly Ser Gly Met Ala Ala
                                                             1

      TGT AAT CTA CCA ATA GTC GAA GGC CCC TGC CGA GCC TTC ATC AAG
      Cys Asn Leu Pro Ile Val Glu Gly Pro Cys Arg Ala Phe Ile Lys
           3'-GAT GGT TAT CAG CTT CCG GGG ACG GCT-5'
                R11E primer            3'-ACG GCT CGG AAG TAG TTC
                                                     Q19K primer
                5                  10                  15

      CTC TGG GCA TTT GAT GCT GTC AAG GGG AAG TGC GTC CTC TTC CCC
      Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro
      GAG ACC CGT-5'
               20                  25                  30

                                      Y46E primer
                                5'-GG AAC AAG TTC GAA TCA GAG AAG
      TAC GGG GGC TGC CAG GGC AAC GGG AAC AAG TTC GAA TCA GAG AAG
      Tyr Gly Gly Cys Gln Gly Asn Gly Asn Lys Phe Glu Ser Glu Lys
               35                  40                  45

      G-3'
      GAG TGC AGA GAG TAC TGC GGT GTC CCT GGT GAT GGT GAT GAG GAG
      Glu Cys Arg Glu Tyr Cys Gly Val Pro Gly Asp Gly Asp Glu Glu
               50                  55                  60

                                      BamHI
      CTG CTG CGC TTC TCC AAC TGA CAACTGGATCCTCTACGCCGGACGCATCGT
      Leu Leu Arg Phe Ser Asn ...       3'- GAGATGCGGCCTGCGTAGCA-5'
               65          68                pBRBamHI primer
```

Fig. 45

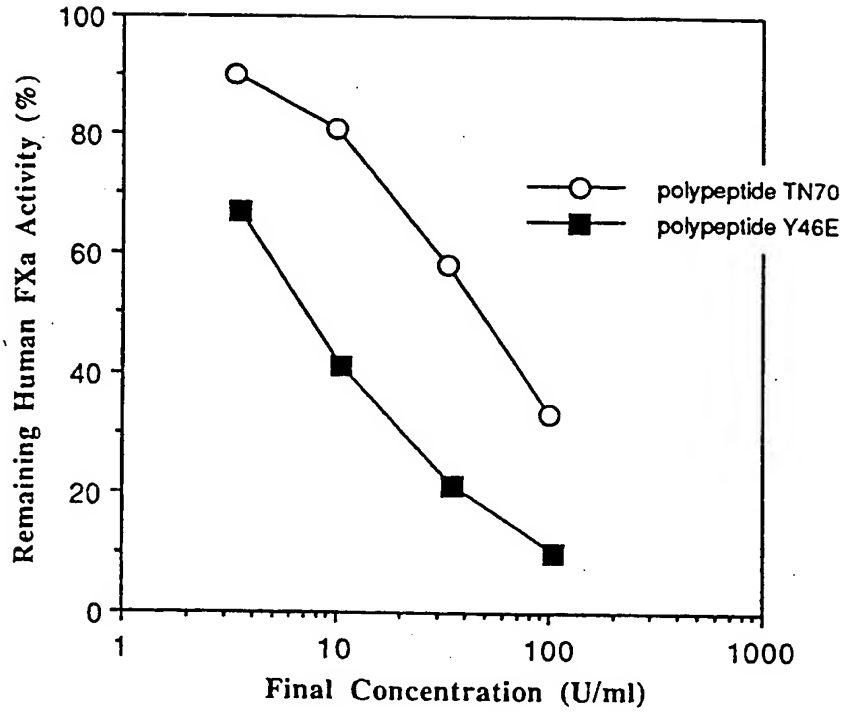


Fig. 46

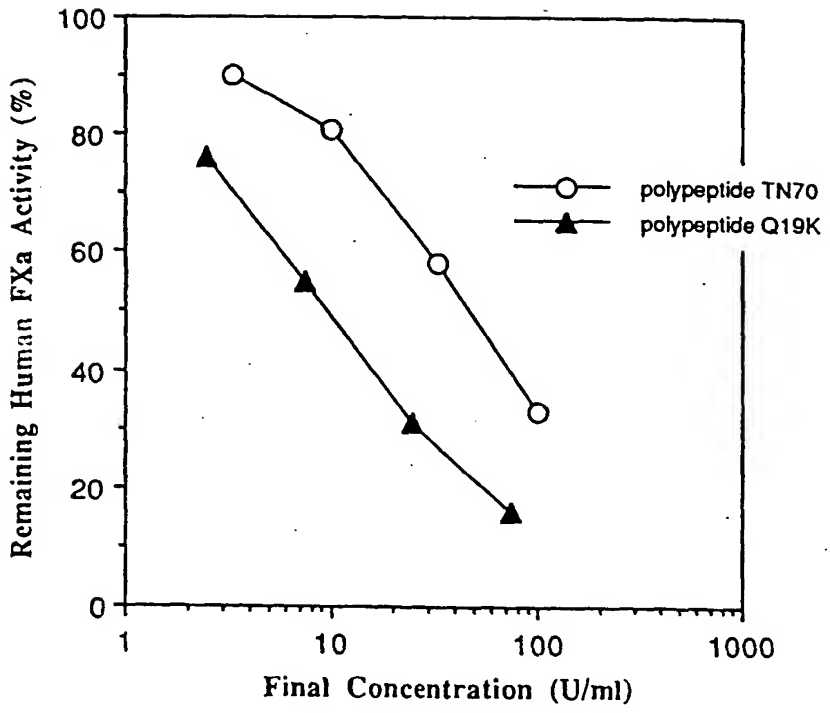


Fig. 47

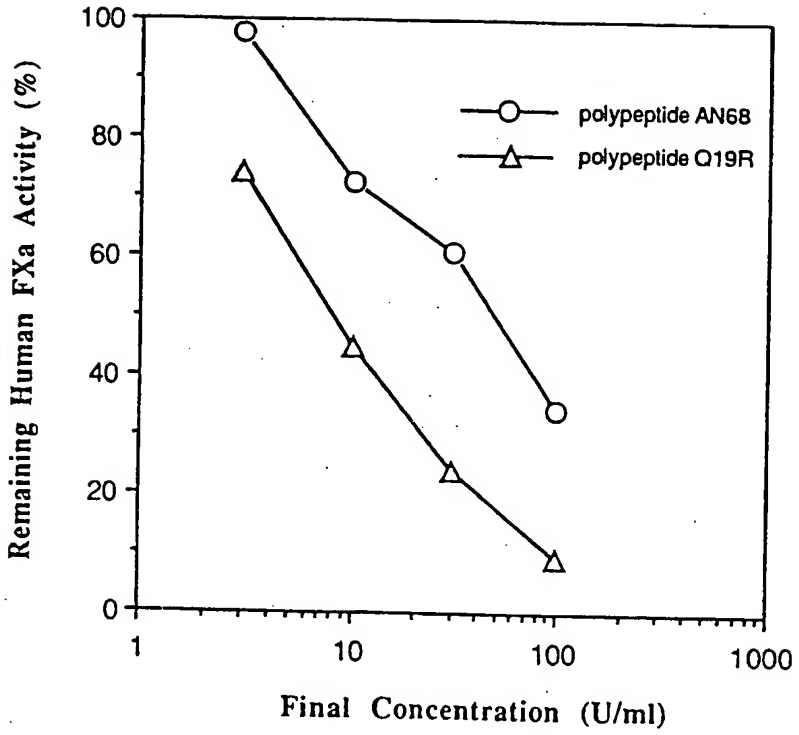


Fig. 48

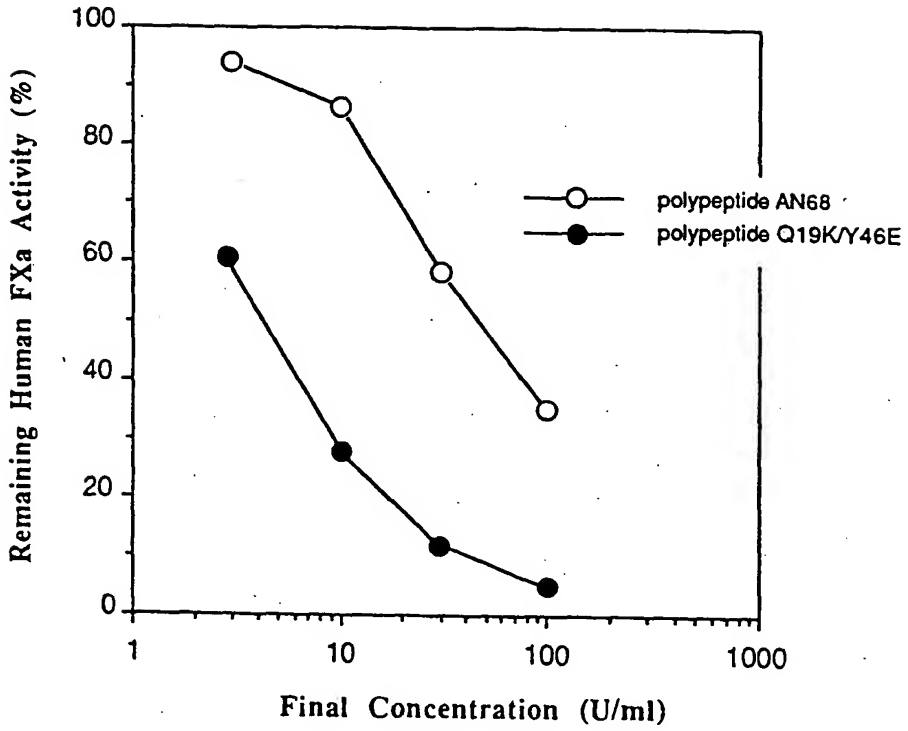


Fig. 49

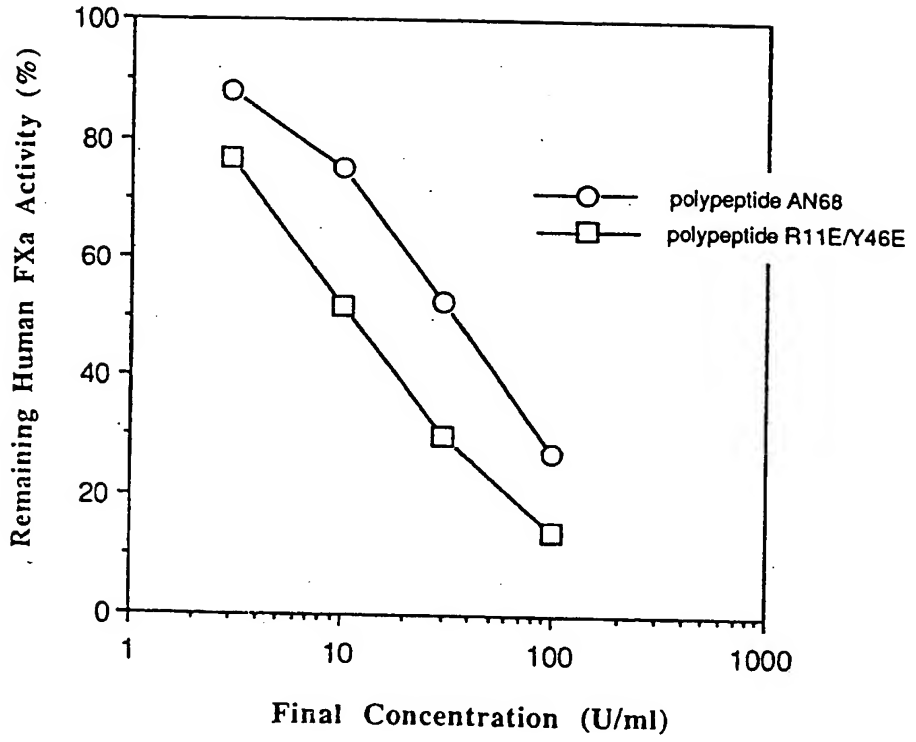


Fig. 50

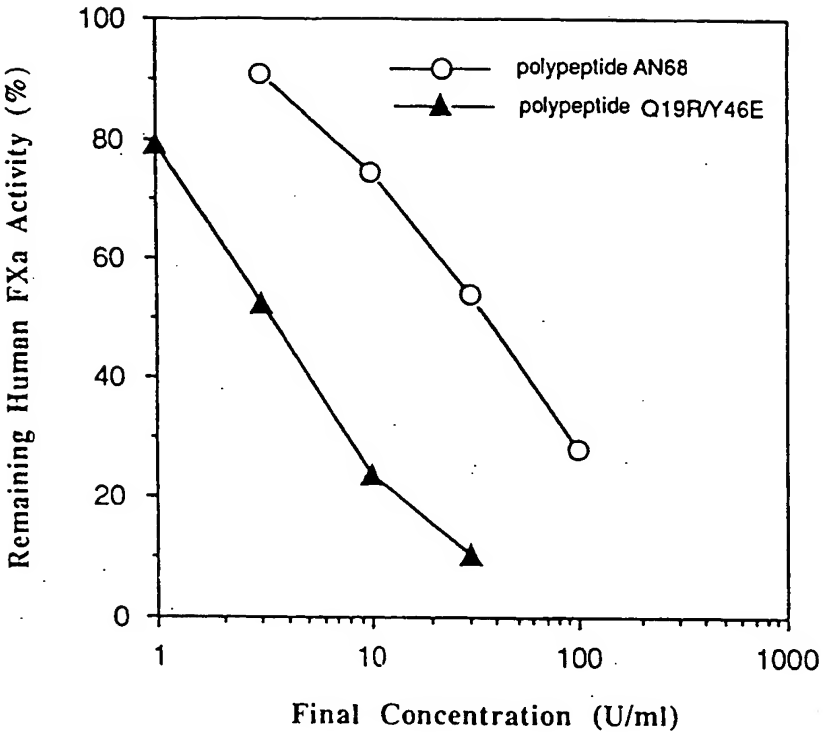

100
80
60
40
20
0
1
10
100
1000
Remaining Human FXa Activity (%)
Final Concentration (U/ml)
polypeptide AN68
polypeptide Q19R/Y46E

Fig. 51

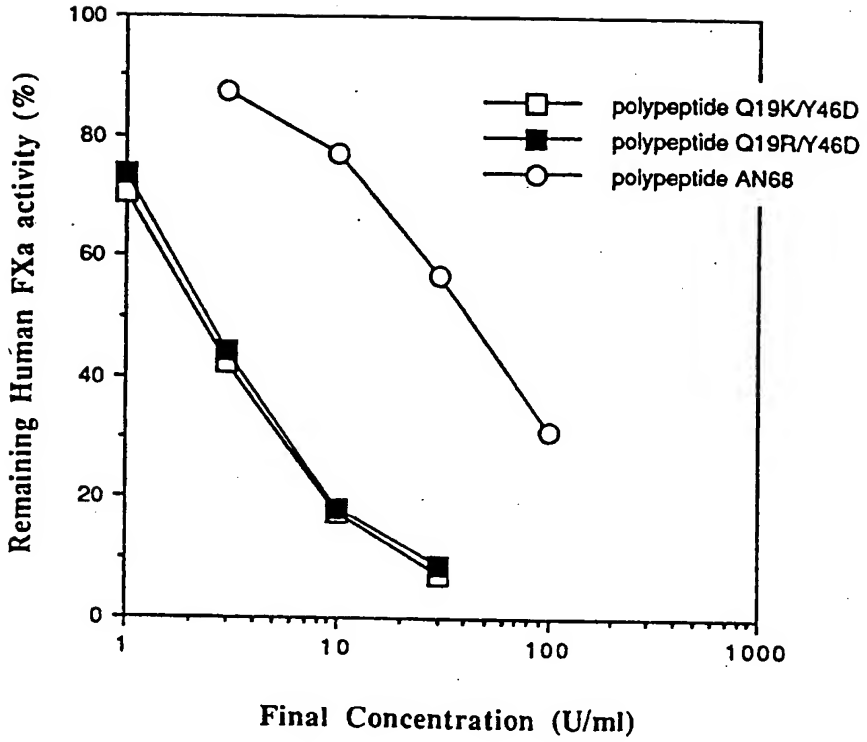


Fig. 52

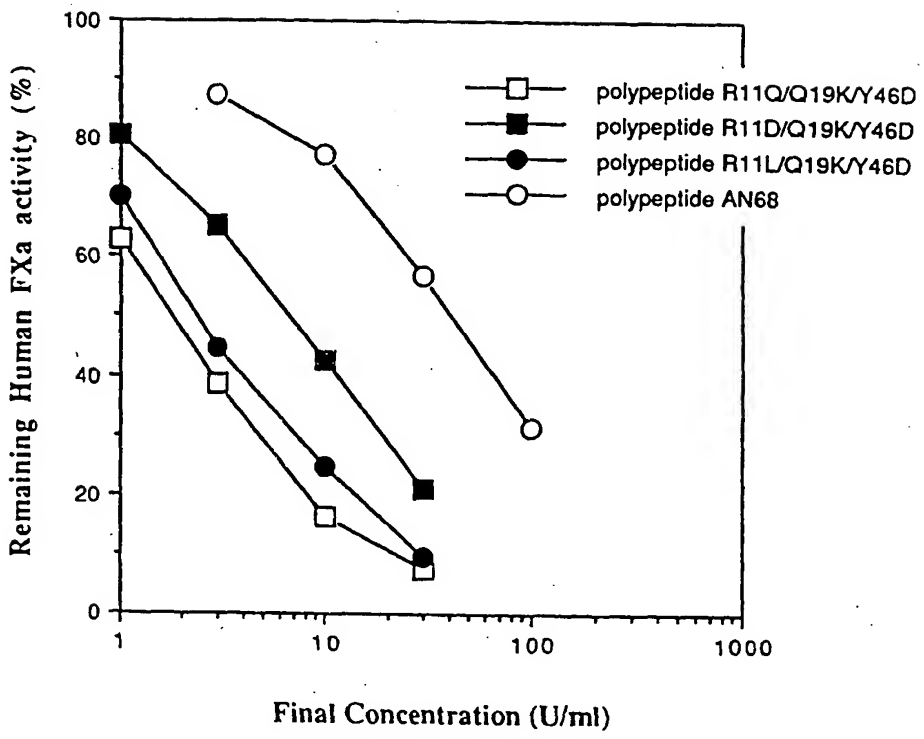


Fig. 53

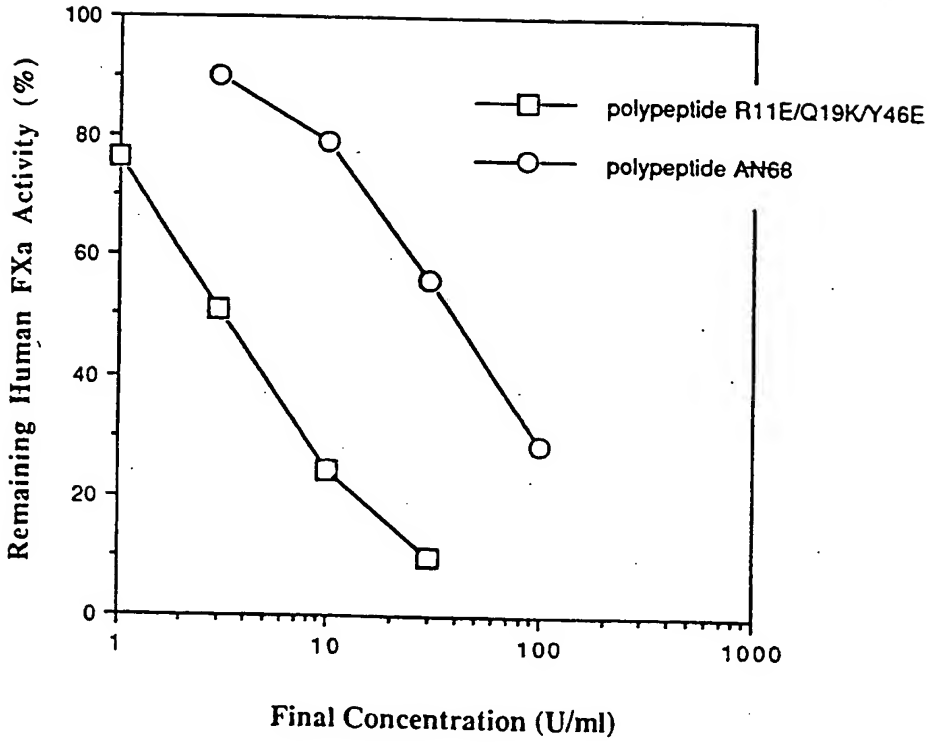


Fig. 54

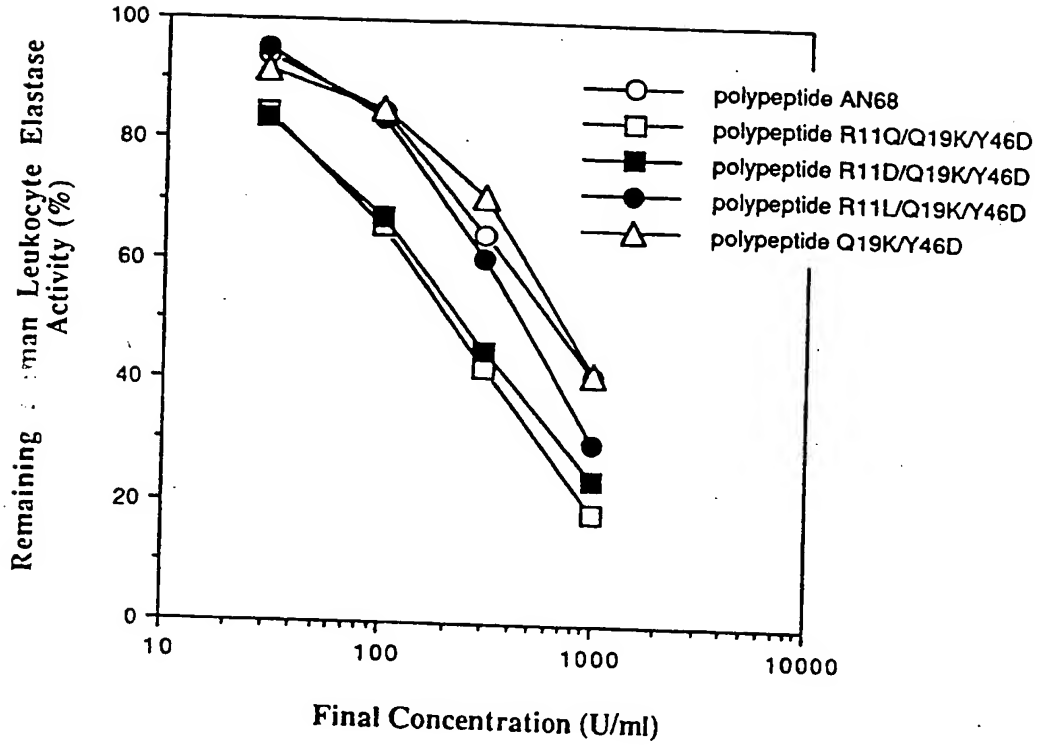
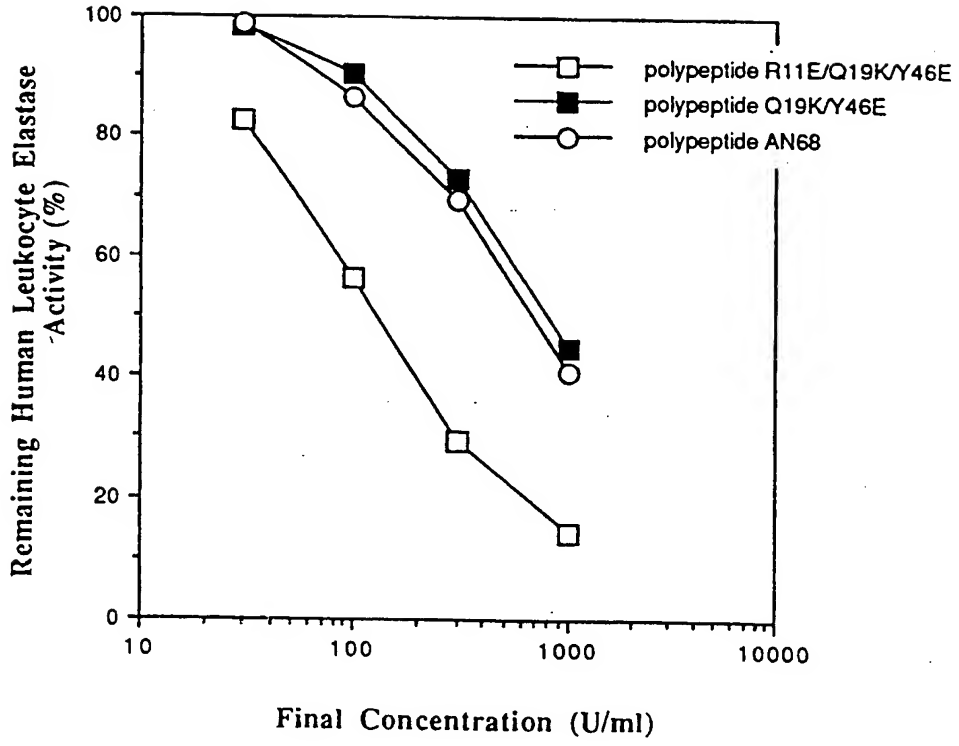

Remaining Human Leukocyte Elastase Activity (%)
100
80
60
40
20
0
10
100
1000
10000
Final Concentration (U/ml)
polypeptide AN68
polypeptide R11Q/Q19K/Y46D
polypeptide R11D/Q19K/Y46D
polypeptide R11L/Q19K/Y46D
polypeptide Q19K/Y46D

Fig. 55


Remaining Human Leukocyte Elastase Activity (%)
100
80
60
40
20
0
10
100
1000
10000
Final Concentration (U/ml)
polypeptide R11E/Q19K/Y46E
polypeptide Q19K/Y46E
polypeptide AN68

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 92 11 9083

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	EP-A-0 486 001 (MOCHIDA PHARMACEUTICAL CO.) 20 May 1992 * claims 5-14,50-58 *	1-5, 7-13, 15-19,22	C12N15/15 C07K13/00 C12P21/02 A61K37/64 C12Q1/37
D,X	EP-A-0 401 508 (BAYER AG) 12 December 1990	1-7,22	
A	* claims 8-10 *	18,19	
A	EP-A-0 073 251 (MOCHIDA PHARMACEUTICAL CO., LTD.) 9 March 1983 * claim 1 *	21	
A	CHEMICAL ABSTRACTS, vol. 107, no. 25, 21 December 1987, Columbus, Ohio, US; abstract no. 228724c, N. SAKURAGAWA ET AL. 'Effect of urinastatin on coagulation, fibrinolysis, and platelet aggregation in vitro and in vivo' page 46 ;column R ; * abstract * & SAISHIN IGAKU vol. 42, no. 4, 1987, OSAKA, JAPAN pages 820 - 830	21	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)
C07K
C12N
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	29 JANUARY 1993	THIELE U.H.-C.H.

CATEGORY OF CITED DOCUMENTS

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